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**14. ABSTRACT**

We show that IL-17 mediated endothelial migration and tube formation is mediate through ligation of IL-17 to IL-17RC and activation of PI3K/AKT pathway. To demonstrate the indirect role of IL-17 in rheumatoid arthritis (RA) angiogenesis, potent proangiogenic factor such as VEGF was examined. Interestingly we found that that RA synovial fluid induced endothelial chemotaxis mediated by IL-17 did not synergize with VEGF suggesting that VEGF may not play a crucial role in IL-17 induced joint neovascularization. Therefore role of other potential proangiogenic factors was examined in IL-17 induced arthritis model. We show that expression of CXCL1 and CXCL5 is highly elevated in RA synovial tissues treated with IL-17 and in IL-17 induced experimental arthritis while VEGF was not markedly increased in any of the mentioned models. Next we document that immunodepletion of CXCL5 but not CXCL1 relieves IL-17 induced arthritis. Results from our study further demonstrate that anti-CXCL5 antibody treatment reduces joint TNF-$\alpha$ levels and vascularization in IL-17 induced arthritis model. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF-$\kappa$B signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF-$\kappa$B mediated angiogenesis through an IL-17 non-overlapping mechanism. In conclusion, these observations suggest that IL-17-mediated joint vascularization may be in part due to CXCL5 induction.

**15. SUBJECT TERMS**

IL-17, angiogenesis, Rheumatoid arthritis, experimental arthritis, VEGF, CXCL1 and CXCL5

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The specific aims of this proposal will remain unchanged and will be performed as initially proposed.


## INTRODUCTION

TH-17 cells are a newly discovered CD4 helper T-cells that produce interleukin-17A (also known as IL-17). IL-17 is found in Rheumatoid Arthritis (RA) synovial tissue and fluid, and the percentage of TH-17 cells is significantly higher in RA synovial fluid compared to RA or normal peripheral blood (1, 2). IL-17 has been shown to have a profound effect in experimental models of arthritis however its role in Rheumatoid Arthritis is undefined. Angiogenesis is an early and a critical event in the pathogenesis of RA. Since our preliminary data suggests that IL-17 plays an important role in RA angiogenesis, this grant was proposed to determine the mechanism by which IL-17 induces neovascularization.

Our overriding hypothesis is that IL-17 mediates angiogenesis in RA through activation of the PI3K pathway, and this effect may be dependent or independent of other proangiogenic factors. To test this hypothesis we will investigate the contribution of IL-17 and its receptors to RA synovial fluid-mediated endothelial migration and blood vessel growth. We will also identify signaling pathways that are involved in IL-17-mediated neovascularization and examine whether mice deficient in a particular signaling pathway are unable to mediate neovascularization through IL-17. Additionally, the indirect role of IL-17 in induction of angiogenesis and arthritis will be examined by blocking the effect of relevant IL-17 associated pro-angiogenic factors in *in vitro* and *in vivo* models of angiogenesis and experimental arthritis. The unmet need is to elucidate the mechanisms by which IL-17 mediates angiogenesis in RA and to determine whether targeting IL-17 and/or its intermediary molecules will provide a therapeutic intervention for RA patients.


## BODY

In **task 1**, we proposed to examine the mechanism by which IL-17 induces angiogenesis, (time frame, months 1-13).

**A summary of the 2011 progress report:** In our progress report submitted in July of 2011 we addressed the specific aims proposed in Task 1 by demonstrating that ligation of IL-17 to IL-17RC but not IL-17 RA is responsible for the direct effect of IL-17 on angiogenesis. We further documented that ligation of IL-17 to IL-17RC mediates endothelial migration and tube formation via activation of AKT1/PI3K pathway. IL-17 expressed in the joint synovial fluid plays a crucial role in RA pathogenesis since neutralization of IL-17 in the fluid or IL-17RC on endothelial cells markedly reduces endothelial homing and vessel formation. *In vivo* we demonstrate for the first time that local expression of IL-17 induces joint inflammation that is associated with increased vascularization. Confirming these observations we also show that hemoglobin concentration is 10 fold higher in IL-17 containing Matigel plugs compared to PBS controls. These results validate that IL-17 can exacerbate RA pathogenesis by directly inducing angiogenesis through ligating to IL-17RC and activating of AKT1 pathway. These results are published in J Immunol 2010, 184:3233-3241. Most recently others have shown that IL-17 is capable of inducing endothelial cell invasion by activating production of CXCL1/GROα and MCP-1 from RA synovial tissue fibroblasts (3) suggesting that there may be other mechanisms involved in IL-17 mediated vascularization. Therefore we next studied the indirect role of IL-17 in RA pathology and angiogenesis proposed in aim 3.

**TASK 2.** To determine whether IL-17 plays a role in endothelial cell homing in RA ST severe combined immunodeficient (SCID) chimera model. We will: **(time frame, months 14-24).**

Task 2 has not been achieved.


**TASK 3 :** To examine the indirect role of IL-17 in mediating angiogenesis and arthritis. We will: **(time frame, months 24-36).**

**3a. examine whether IL-17-induced angiogenesis will be suppressed by blocking VEGF. (time frame, months 24-26).**

To accomplish this aim, we initially examined whether IL-17 and VEGF expressed in RA synovial fluid have a synergistic effect on RA synovial fluid mediated angiogenesis. We found that immunodepletion of IL-17 and VEGF in RA synovial fluids does not have an additive or synergistic effect in reduction of human vascular endothelial cells (HMVEC) migration beyond the effect noted with neutralization of one factor alone (Fig. 1) suggesting that both IL-17 and VEGF may be mediating HMVEC migration through the same signaling pathway.
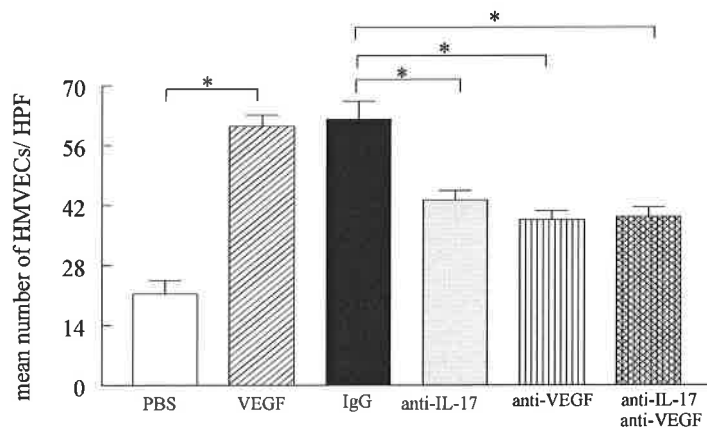


**Figure 1. IL-17 and VEGF present in RA synovial fluid do not synergize in mediating endothelial migration.** RA synovial fluids from 8 patients (1:20 dilution) were incubated with antibodies to IL-17 (10 µg/ml), VEGF (10 µg/ml), or both as well as isotype control or PBS or VEGF for 1h prior to performing HMVEC chemotaxis in response to RA synovial fluids. The values represent the mean ± SE. * represents p <0.05.


In order to determine that IL-17 modulates proangiogenic factors in RA synovial tissue explants and IL-17-induced arthritis model, levels of CXCL1, CXCL5, FGF2 and VEGF were quantified in IL-17 activated RA synovial tissue explants and/or IL-17-mediated arthritis ankles (harvested from day 10 post injection) and the data was demonstrated as fold increase above the control group (Figs. 2A-B). The results obtained from IL-17-induced arthritis model are similar to our finding in RA synovial tissue explants in that CXCL1 and CXCL5 are induced to a greater extent (40-10 fold increase in IL-17-induced arthritis ankles and 7-12 fold increase in RA explants compared to the control group) compared to FGF2 (3-2 fold increase respectively) while VEGF was not significantly elevated in any of the mentioned models (fig. 2A-B). Although in the IL-17-induced arthritis model the fold increase levels above Ad-control is greater for CXCL1 (40 fold) compared to CXCL5 (10 fold) the absolute joint concentrations for CXCL1 (1600 pg/ml) and CXCL5 (1520 pg/ml) are comparable on day 10 post injection (Figs. 2C-D). Based on these results we concluded that CXCL1 and CXCL5 may be important in IL-17 mediated pathogenesis in RA and experimental arthritis model.
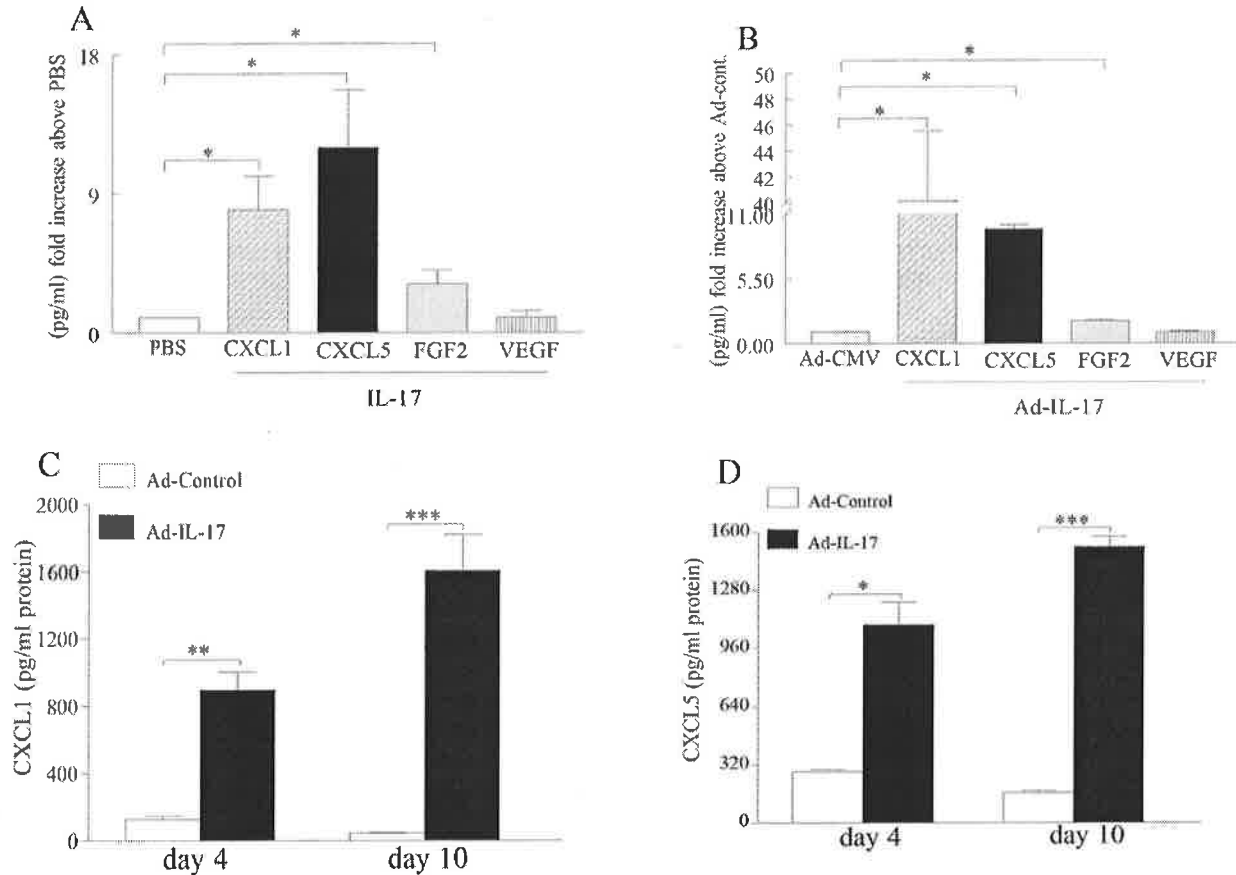
**Figure 2. IL-17 increases the expression of CXCL1 and CXCL5 in RA synovial tissue explants and experimental arthritis model. A.** RA synovial tissue explants were treated with PBS or IL-17 (100 ng/ml), tissues were harvested after 24h and levels of CXCL1, CXCL5, FGF2 and VEGF were quantified by ELISA and normalized to PBS values. **B.** Ad-IL-17 or Ad-CMV control ($10^7$ PFU) was injected intra-articularly into C57/BL6 mice. Ankles were harvested on day 10 and levels of CXCL1, CXCL5, FGF2 and VEGF were measured by ELISA and normalized to Ad-CMV. **C.** Ankles from Ad-IL-17 and Ad-CMV treatment were harvested on days 4 and 10, and CXCL1 **(C)** and CXCL5 **(B)** levels were quantified by ELISA. Values are reported as mean ± SE. * denotes p<0.05, ** denotes p<0.01 and *** denotes p<0.005, n=8-10.

**3b and 3c. Investigate whether IL-17 angiogenesis *in vivo* is due to down stream proangiogenic factors. (time frame, months 26-36).**

*Inhibition of CXCL5 but not CXCL1 ameliorates IL-17-induced arthritis.*

Next experiments were performed to determine whether CXCL1 and/or CXCL5 play a role in arthritis mediated by local IL-17 expression in mice ankle joints. In mice locally injected with IL-17 (and IgG control), disease activity determined by ankle circumference began around day 3 and progressed through day 5, plateauing thereafter until the termination of the experiments on day 10 (Fig. 3A). The disease activity determined by ankle circumference was significantly lower in mice receiving anti-CXCL1 on days 3 and 5, compared to the control group. However, as the arthritis progressed there was no difference noted at later time points (days 7 and 9) (Fig. 3A). In vitro chemotaxis performed on

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endothelial cells demonstrated that the anti-CXCL1 antibody could markedly suppress CXCL1-induced endothelial migration suggesting the functionality of anti-CXCL1 antibody (data not shown). Further, mice receiving anti-CXCL5 demonstrated significantly reduced clinical signs of arthritis at all time points, compared to the mice treated with IgG control (p<0.05). The combination of anti-CXCL1 and anti-CXCL5 did not ameliorate IL-17-induced joint inflammation beyond the effect observed using anti-CXCL5 alone (Figs. 3A-C). Next, histological examination of the joints was performed to determine the effect of treatment on inflammation, synovial lining and joint destruction. Consistent with the clinical data, histological analysis of the treatment groups demonstrated that inflammation, synovial lining thickening, and bone erosion were markedly reduced in the anti-CXCL5 and anti-CXCL1 and anti-CXCL5 treatment groups. In contrast, mice receiving anti-CXCL1 antibody had similar clinical scores compared to the control group (Figs. 3B and C). Our results demonstrate that CXCL5, and not CXCL1, plays an important role in IL-17-mediated arthritis.
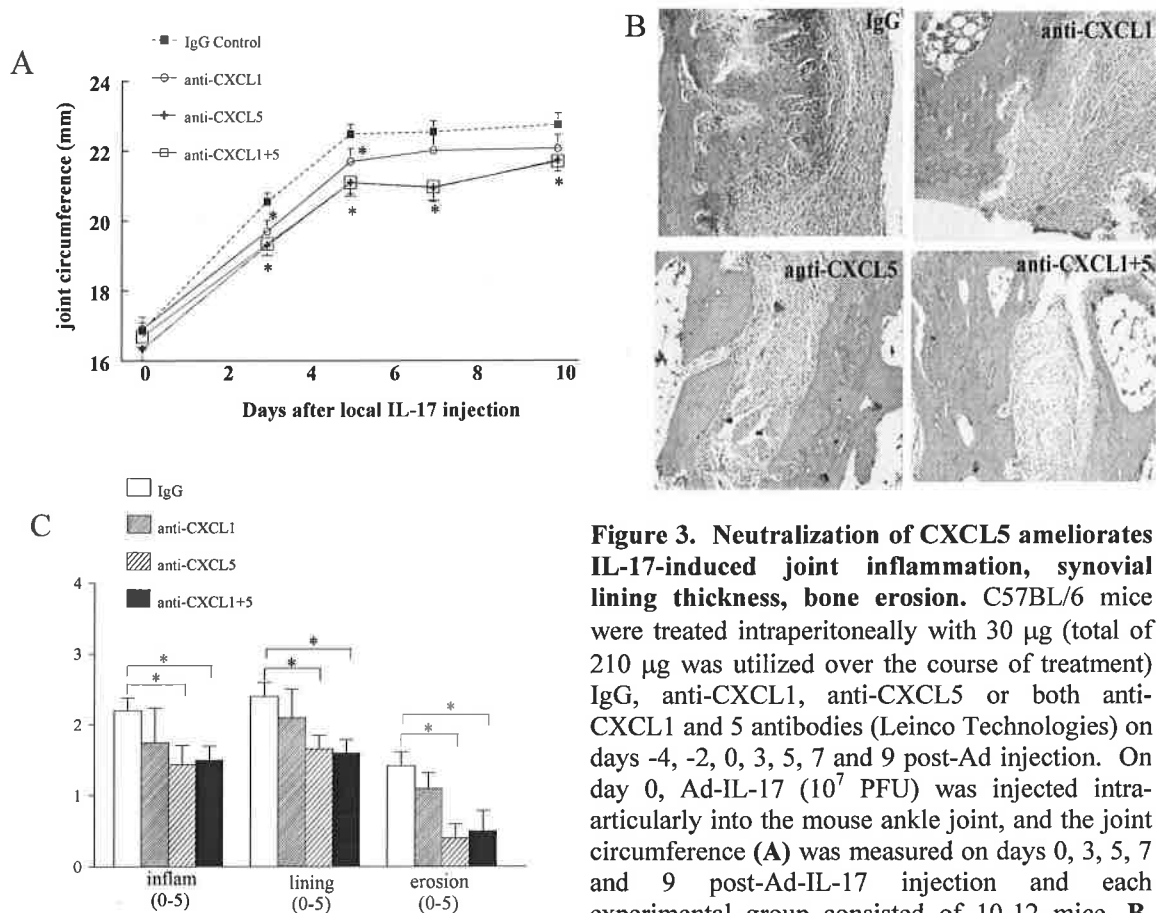


Figure 3. Neutralization of CXCL5 ameliorates IL-17-induced joint inflammation, synovial lining thickness, bone erosion. C57BL/6 mice were treated intraperitoneally with 30 µg (total of 210 µg was utilized over the course of treatment) IgG, anti-CXCL1, anti-CXCL5 or both anti-CXCL1 and 5 antibodies (Leinco Technologies) on days -4, -2, 0, 3, 5, 7 and 9 post-Ad injection. On day 0, Ad-IL-17 ($10^7$ PFU) was injected intra-articularly into the mouse ankle joint, and the joint circumference (A) was measured on days 0, 3, 5, 7 and 9 post-Ad-IL-17 injection and each experimental group consisted of 10-12 mice. B. and C. Inflammation, synovial lining and bone erosion (based on a 0–5 score) were determined using H&E-stained sections by a blinded observer, n=10 ankles. Values demonstrate mean ± SE. * denotes p<0.05.

*Anti-CXCL5 treatment downregulates proinflammatory mediators in IL-17-induced arthritis model.*
To determine the role of CXCL1 and CXCL5 on IL-17-induced arthritis, proinflammatory mediators were quantified in ankle joints. For this purpose, the effect of therapy was examined on joint TNF-α, IL-6, IL-1β, CCL2, CCL3, CCL5, CCL20, CXCL2, FGF2 and VEGF protein levels. Our results demonstrate that mice receiving anti-CXCL5 or combination therapy had 40-50% lower levels of joint TNF-α, compared to the control group (Fig. 4A). Joint CCL5 levels were also significantly (40-50%)

reduced in IL-17-induced arthritis ankles receiving anti-CXCL5 or combination of anti-CXCL1 and 5, respectively (data not shown). Other joint proinflammatory mediators such as IL-6, IL-1β, CCL2, CCL3, CCL20, and CXCL2 were not affected by anti-CXCL5, anti-CXCL1 or combination treatments (data not shown). To demonstrate that the efficacy of anti-CXCL5 treatment is independent of reduction potent proangiogenic factors, joint FGF2 and VEGF were quantified in all four treatment groups. The data demonstrate that although levels of FGF2 but not VEGF are elevated in IL-17-induced arthritis model, ankles treated with anti-CXCL1 and anti-CXCL5 have similar levels of FGF2 (Fig. 4B) and VEGF (Fig. 4C) suggesting that anti-CXCL5 treatment can directly suppress IL-17-mediated angiogenesis independent of FGF2 or VEGF pathways. Reduction in joint TNF-α levels in the anti-CXCL5 and combination therapy may be due to the fact that IL-17-induced joint pathology is abrogated in TNF-α deficient mice, indicating that in this model TNF-α is required (4). It has also been shown that IL-17 can directly modulate TNF-α secretion from macrophages (5) hence, suppressing IL-17-induced inflammation may reduce TNF-α production from macrophages in the synovial lining and sublining. Further, both TNF-α and IL-17 synergize in inducing the expression of CXCL5 from RA fibroblasts (data not shown). Therefore, neutralization of CXCL5 may have a negative feed back regulation on joint TNF-α concentrations. When RA synovial tissue fibroblasts, macrophages and HMVECs were screened for IL-17 downstream targets, CCL5 was undetected (data not shown). Therefore, reduction in joint CCL5 concentration in anti-CXCL5 and combination therapy treatment groups may be due to reduced TNF-α levels, since CCL5 expression is known to be modulated by TNF-α in RA synovial tissue fibroblasts (6, 7).
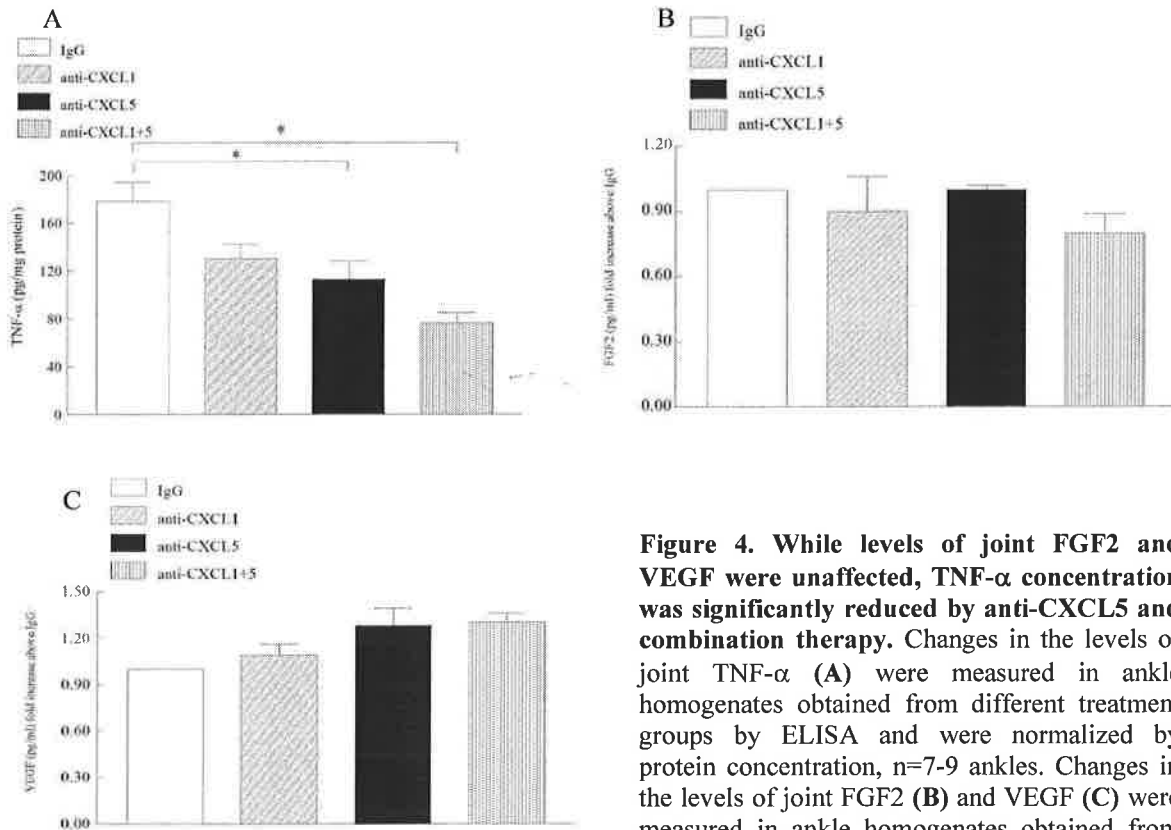


Figure 4. While levels of joint FGF2 and VEGF were unaffected, TNF-α concentration was significantly reduced by anti-CXCL5 and combination therapy. Changes in the levels of joint TNF-α (A) were measured in ankle homogenates obtained from different treatment groups by ELISA and were normalized by protein concentration, n=7-9 ankles. Changes in the levels of joint FGF2 (B) and VEGF (C) were measured in ankle homogenates obtained from different treatment groups by ELISA and the data is presented as fold increase above the control IgG treatment, n=7-9 ankles. Values demonstrate mean ± SE. * denotes p<0.05.

*Anti-CXCL5 treatment reduces IL-17-induced vascularization.*

To determine the mechanism by which anti-CXCL5 ameliorates IL-17-induced arthritis, ankles were examined for joint vascularization. The data demonstrate that while levels of vascularization were similar in the IgG and the anti-CXCL1 treatment groups, anti-CXCL5 and the combination therapy had 40% fewer blood vessels (Figs 5A and B). Our results may suggest that neutralization of CXCL5 can affect IL-17-induced arthritis through reduced blood vessel formation.
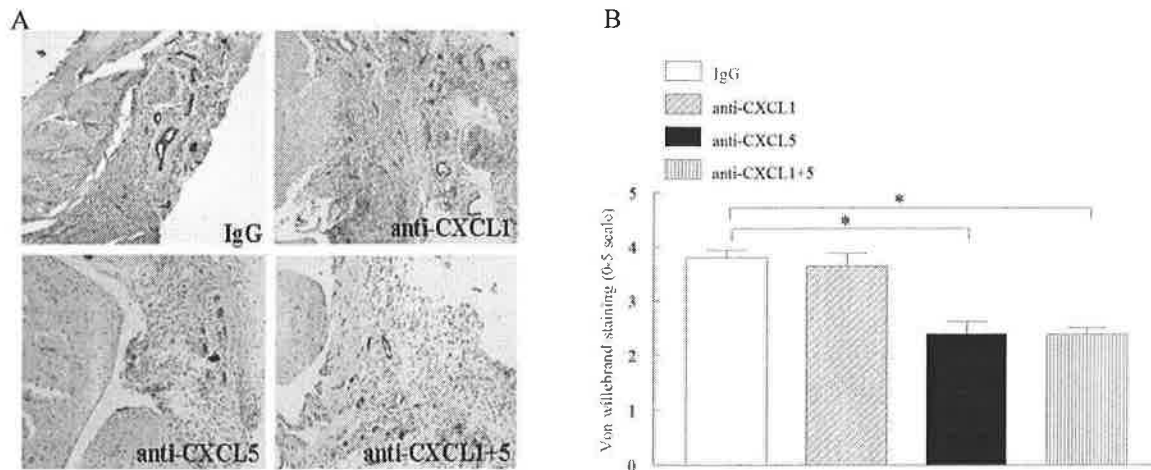


**Figure 5. Anti-CXCL5 therapy downregulates IL-17-induced joint vascularization.** IL-17-induced arthritis ankles treated with IgG, anti-CXCL1, anti-CXCL5 or the combination therapy were harvested on day 11 and were immunostained with Von willebrand factor (endothelial marker) (**A**) (original magnification x 200). Quantification of endothelial (**B**) staining from IL-17-induced arthritis ankles harvested on day 11, n=8-9 ankles. Values demonstrate mean ± SE. * denotes $p<0.05$.

*Number of blood leukocytes, neutrophils and monocytes were unaffected in anti-CXCL1 and 5 treatments.*

To determine whether the IL-17-induced arthritis model could be affected by systemic treatment with anti-CXCL1 and/or anti-CXCL5, the number of leukocytes, neutrophils and monocytes were measured in mouse whole blood. Interestingly, all treatment groups had similar numbers of white blood cells, neutrophils and monocytes (Fig. 6), in contrast to lower levels of joint neutrophils in the anti-CXCL5 and combination therapy (data not shown). These results suggest that although the number of circulating cells was unchanged in the anti-CXCL5 and combination therapy groups, fewer cells migrated into these IL-17-mediated arthritis joints (as shown in H&E staining in Figs. 3B and C).

| Cell type | IgG | anti-CXCL1 | anti-CXCL5 | anti-CXCL1+5 |
|---|---|---|---|---|
| Leukocytes k/μl | 11.30±1.14 | 11.44±0.68 | 10.29±0.67 | 11.73±0.8 |
| Neutrophils k/μl | 1.58±0.18 | 1.61±0.13 | 1.38±0.11 | 1.46±0.17 |
| Monocytes k/μl | 0.42±0.04 | 0.38±0.03 | 0.43±0.04 | 0.49±0.03 |

**Figure 6. Anti-CXCL5 treatment did not affect the circulating number of leukocytes, neutrophils and monocytes in IL-17-mediated arthritis model.** On day 11 blood was collected by cardiac puncture of IL-17-induced arthritis ankles treated with IgG, anti-CXCL1, anti-CXCL5 or the

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combination therapy to measure blood cell count using a HemaVet 850 complete blood counter. Values are shown in thousands of cells per microliter of blood (k/μl, n=10-12 mice).

***CXCL1 and CXCL5 mediate endothelial migration through CXCR2 ligation.*** To demonstrate whether CXCR2 ligation is involved in CXCL1 and CXCL5 function, CXCR2 on endothelial cells was blocked employing anti-CXCR2 neutralizing antibody and subsequently endothelial chemotaxis was examined in response to CXCL1 and CXCL5. Results from these experiments demonstrate that neutralization of CXCR2 on HMVECs significantly decreases CXCL1 (50%) and CXCL5 (40-50%) -mediated endothelial migration (Fig. 7) suggesting that both chemokines require CXCR2 ligation in order to mediate chemotaxis despite them signaling through different signaling pathways.
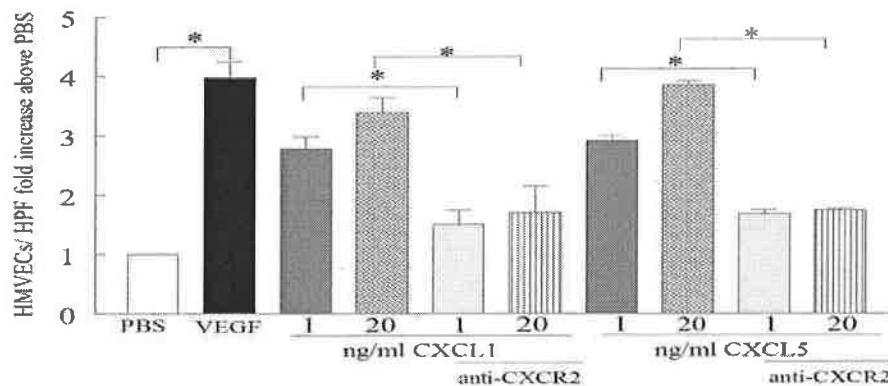


**Figure 7. CXCL1 and CXCL5 mediate endothelial migration through CXCR2 ligation.** Human endothelial microvascular cells (HMVEC)s incubated with antibody to CXCR2 (10 μg/ml, R&D systems) were kept at 37°C for 2h while cells were attached to the membrane and chemotaxis was examined in response to CXCL1 and CXCL5 (1 and 20 ng/ml; for 2h at 37°C), n=2. Values represent fold increase chemotaxis above cells migrating in response to PBS shown as mean ± SE of two experiments in triplicate. * represents p <0.05.

***CXCL1 and CXCL5 induce endothelial migration through different signaling pathways.***
To address the different efficacy of blocking CXCL1 and CXCL5 in IL-17 experimental arthritis model we examined the mechanism by which these chemokines induce endothelial migration. We found that in HMVECs, CXCL1 signals through PI3K (35 min) and ERK (35 min) however this chemokine was unable to activate NF-κB or p38 signaling pathways (Figs. 8A). CXCL5 stimulation of HMVECs results in activation of NF-κB pathway (65 min) only (Figs. 8C). To demonstrate the mechanism by which CXCL1 and CXCL5 mediate HMVEC migration, inhibitors to these pathways were employed in *in vitro* chemotaxis. Interestingly while inhibition of PI3K suppresses CXCL1-induced HMVEC migration, chemotaxis mediated by CXCL5 was reduced through NF-κB inhibition (Figs. 8B and D). These results suggest that endothelial migration is differentially regulated by CXCL1 and CXCL5.

Other studies have shown that while stimulation with CXCL1 can phosphorylate ERK1/2 pathway (8-10), activation with CXCL5 is involved with PI3K and NF-κB signaling pathways (11). We further demonstrate that similar to IL-17 (12), CXCL1 mediated HMVEC migration is through PI3K activation. In contrast, inhibition of NF-κB suppresses endothelial chemotaxis induced by CXCL5. Perhaps inhibition of CXCL1 is ineffective in reducing joint inflammation since IL-17 is present in the mouse ankles (1200 pg/mg and 400 pg/mg on days 4 and 10 post injection respectively (13)) and can

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induce angiogenesis through the same mechanism. In line with our finding others have shown that CXCL1 and 5 can differentially modulate monocyte arrest and migration (14), suggesting that ligands binding to the same receptor can have distinct functions through activating different signaling intermediates. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF-κB signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF-κB mediated angiogenesis through an IL-17 nonoverlapping mechanism. In conclusion, these observations suggest that IL-17-mediated joint vascularization may be in part due to CXCL5 induction.
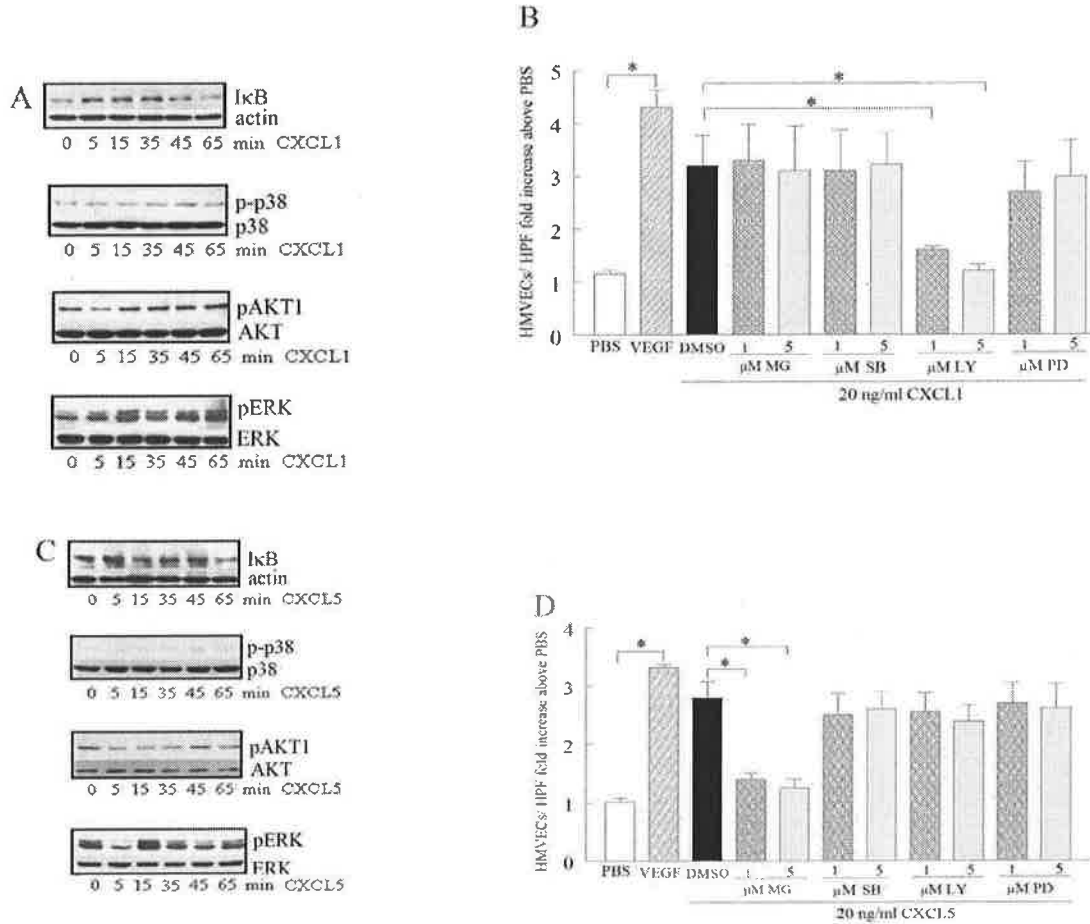


**Figure 8. CXCL1 and CXCL5 induce endothelial migration through activating different signaling pathways.** In order to determine the mechanism by which **(A)** CXCL1 and **(C)** CXCL5 activate HMVECs, cells were stimulated with these chemokines (20 ng/ml) for 0-65 minutes, and the cell lysates were probed for IκB, p-p38, p-AKT ,and pERK and/ or equal loading controls. To determine signaling pathways associated with **(B)** CXCL1 and **(D)** CXCL5-induced HMVEC migration, cells were treated with DMSO or inhibitors to NF-κB (MG-132; 1 and 5 μM), p38 (SB203580; 1 and 5 μM), PI3K (LY294002; 1 and 5 μM) and ERK (PD98059; 1 and 5 μM) 2h in the Boyden chamber, n=2. Values represent fold increase chemotaxis above cells migrating in response to PBS shown as mean ± SE of two experiments in triplicate. * represents p <0.05.

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## KEY RESEARCH ACCOMPLISHMENTS

Within this short time frame we have shown that:

- RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 and IL-17 does not synergize with VEGF in RA synovial fluid induced HMVEC migration.

- Neutralization of IL-17RC but not IL-17RA was involved with suppressing RA synovial fluid-mediated HMVEC migration.

- Activation of PI3K is responsible for IL-17-mediated HMVEC tube formation and migration.
- Expression of CXCL1 and CXCL5 is highly elevated in RA synovial tissues treated with IL-17 and in IL-17 induced arthritis model while VEGF was not markedly increased in any of the mentioned models.
- Inhibition of CXCL5 but not CXCL1 relieves IL-17-induced arthritis.
- Anti-CXCL5 reduces levels of joint TNF-$\alpha$ and vascularization in IL-17-induced arthritis model.
- CXCL5 but not CXCL1 induces endothelial migration and angiogenesis through an IL-17 non overlapping mechanism.
- IL-17 mediated joint vascularization is in part due to CXCL5 induction.

## REPORTABLE OUTCOMES

**Publications:**

1. Pickens SR, Volin MV, Mandelin II AM, Kolls JK, Pope RM, **Shahrara S**. IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis. J. Immunol. 184; 6 :3233-3241, 2010.

2. **Shahrara S**, Pickens SR, Mandelin AM, Huang Q, Karpus W, Kolls JK, Pope RM. IL-17-mediated monocyte migration is partially through CCL2/MCP-1 induction. J. Immunol. 184; 8:4479-4487, 2010.

3. Ruth RH, Park CC, Amin MA, Lesch C, Marotte H, **Shahrara S** and Koch AE. IL-18 as an *in vivo* Mediator of Monocyte Recruitment in Rodent Models of Rheumatoid Arthritis. Arthritis Res. Ther. 16; 12: R118, 2010.

4. Thumbikat P, **Shahrara S**, Sobkoviak R, Done J, Pope RM and Schaeffer AJ. Prostate secretions from men with CP/CPPS inhibits proinflammatory mediators. J. Urol. 184:1536-42, 2010.

5. Pickens SR, Chamberlain ND, Volin MV, Pope RM, Mandelin II AM, *****Shahrara S**: Characterization of CCL19 and CCL21 in rheumatoid arthritis: Arthritis Rheum 63: 914–922, 2011.

6. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM 2nd, Agrawal H, Matsui M, Yoshimoto T, *****Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis: Arthritis Rheum 2011, 63:2289-98.

**Editorial commented on our paper. Fearon U. "IL-27: a master regulator of inflammation" Arthritis Rheum 2011, 63:2157-2160.**

7. *****Shahrara S**. Reply to the Editor. Arthritis Rheum. 2011, 63:2832-2833.

8. Picken SR, Chamberlain ND, Volin MV, Pope RM, Talarico NE, Mandelin AM II and *Shahrara S. Characterization of IL-7 and IL-7R in the pathogenesis of Rheumatoid Arthritis. Arthritis Rheum. 2011, 63:2884-2893.

9. Pickens SR, Chamberlain ND, Volin MV, Gonzalez M, Pope RM, Mandelin AM II, Kolls JK, *Shahrara S. Anti-CXCL5 therapy ameliorates IL-17-induced arthritis by decreasing joint vascularization. Angiogenesis. 2011,14:443-455.

10. Chamberlain ND, Kim SJ, *Shahrara S. Reply to Editor. Arthritis Rheum. 2012, 64:594-595.

11. Pickens SR, Chamberlain ND, Volin MV, Pope RM, Talarico NE, Mandelin AM II, *Shahrara S. Role of the CCL21 and CCR7 pathway in rheumatoid arthritis angiogenesis. Arthritis Rheum. 2012, In press.

12. Chamberlain ND, Vila OM, Volin MV, Volkov S, Pope RM, Swedler W, Mandelin AM 2nd, *Shahrara S. TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF-α Levels. J Immunol. 2012; 189: 475-83.

13. Chamberlain ND, Kim SJ. Vila OM, Volin MV, Volkov S, Pope RM, Arami S, Mandelin AM II, *Shahrara S. Ligation of TLR7 by rheumatoid arthritis synovial fluid single strand RNA induces transcription of TNF-α in monocytes. Annals Rheum Dis. 2012, In press.

14. IL-12 family cytokines in the pathogenesis and treatment of Rheumatoid Arthritis. Pope RM and *Shahrara S. Nature Rev Rheum 2012, In press.

15. Volin MV and *Shahrara S. Role of TH-17 Cells in Rheumatic and Other Autoimmune Diseases. J Rheum 2012. In press.


**Invited Lectures:**

| | |
|---|---|
| 2010 | University of Illinois in Chicago, Rheumatology rounds |
| 2010 | American College of Rheumatology Research and Education Foundation *Within Our Reach* meeting in Dallas |
| 2010 | Northwestern University, Feinberg School of Medicine, Pathology and inflammation group |
| 2010 | American College of Rheumatology 2010 annual meeting held in Atlanta |
| 2011 | Department of Microbiology and Immunology lectures in University of Illinois at Chicago |
| 2011 | The Institute for Personalized Respiratory Medicine lectures in University of Illinois at Chicago |
| 2011 | Department of Rheumatology Grand Rounds in University of Illinois at Chicago |
| 2011 | American College of Rheumatology 2011 annual meeting held in Chicago |

| 2012 | Department of Microbiology and Immunology lectures in University of Illinois at Chicago |
|---|---|
| 2012 | Department of Rheumatology Grand Rounds in University of Illinois at Chicago |

**Abstracts and/or oral presentations presented at American College of Rheumatology (ACR) 2010, 2011 and 2012:**

1. Pickens SR, Volin MV, Mandelin II AM, **Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis. Arthritis Rheum 714: S298, 2010. **Oral presentation**

2. Pickens SR, Volin MV, Pope RM, Mandelin II AM, **Shahrara S**. Characterization of CCR7 and its ligands CCL19 and CCL21 in rheumatoid arthritis. Arthritis Rheum 1506: S627, 2010.

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**Submitted abstracts and/or oral presentations for American College of Rheumatology (ACR) 2012:**

1.Chamberlain ND, Kim SJ, Volin MV, Mehta A, Sweiss N, **Shahrara S**. IL-7; An Important Pro-inflammatory Factor that affects myeloid cell function in RA and CIA. Submitted ACR 2012.

2. Chamberlain ND, Volin MV, Vila OM, Swedler W, Arami S, Volkov S, **Shahrara S**. Expression of TLR5 strongly correlates with levels of TNF-$\alpha$ and DAS28 in RA monocytes and ligation of TLR5 induces angiogenesis in RA. Submitted ACR 2012.

3. Chamberlain ND, Kim SJ, Volin MV, Swedler W, Vila OM, Volkov S, and **Shahrara S**. TLR7 Ligation Contributes to Monocyte Migration in Rheumatoid Arthritis. Submitted ACR 2012.

**Employment opportunities:**

## CONCLUSION

In RA patients percent TH-17 cells correlate with disease activity score suggesting that IL-17 can mediate inflammation at early disease stage and may also be involved in disease progression (15). Therefore to evaluate the role of IL-17 in RA pathogenesis, we examined which IL-17 receptors and signaling pathways are associated with rheumatoid arthritis synovial fluid mediated endothelial migration and tube formation. We show that although HMVECs express both IL-17RA and RC, RA synovial fluid-mediated HMVEC chemotaxis is mediated by binding primarily to IL-17RC. We also report that while IL-17 activates ERK, JNK and PI3K pathways in HMVECs, only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation. We show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone.

To demonstrate whether there are indirect pathways associated with IL-17 induced angiogenesis, we examined potent proangiogenic factors induced by IL-17 in RA synovial tissue as well as in IL-17 experimental arthritis model. We found that expression of CXCL1 and CXCL5 but not VEGF was highly elevated by IL-17 in RA synovial tissue explants and animal models of RA. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17 mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17 mediated disease activity or neovascularization, while being capable of inhibiting CXCL1 mediated endothelial chemotaxis *in vitro*. The combination of anti-CXCL1 and anti-CXCL5 was not more effective than anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5 induced endothelial migration although down stream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF-κB signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF-κB mediated angiogenesis. In conclusion, these observations suggest that IL-17 mediated joint vascularization may be in part due to CXCL5 induction.

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**APPENDICES**

1. Our paper published in J Immunol, 2010, 184:3233-3241, supports our finding presented in this progress report.

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# IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis

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Angiogenesis is an early and a critical event in the pathogenesis of rheumatoid arthritis (RA). Neovascularization is dependent on endothelial cell activation, migration and proliferation, and inhibition of angiogenesis may provide a novel therapeutic approach in RA. In this study, we document a novel role of IL-17 in mediating angiogenesis. Local expression of IL-17 in mouse ankles increases vascularity. We further demonstrate that IL-17 is angiogenic by showing its ability to promote blood vessel growth in Matrigel plugs in vivo. Additionally, IL-17, in concentrations present in the RA joint, induces human lung microvascular endothelial cell (HMVEC) migration mediated through the PI3K/AKT1 pathway. Furthermore, suppression of the PI3K pathway markedly reduces IL-17–induced tube formation. We also show that both IL-17–induced HMVEC chemotaxis and tube formation are mediated primarily through IL-17 receptor C. Neutralization of either IL-17 in RA synovial fluids or IL-17 receptor C on HMVECs significantly reduces the induction of HMVEC migration by RA synovial fluid. Finally, RA synovial fluid immunoneutralized with anti–IL-17 and antivascular endothelial growth factor does not reduce HMVEC migration beyond the effect detected by immunodepleting each factor alone. These observations identify a novel function for IL-17 as an angiogenic mediator in RA, supporting IL-17 as a therapeutic target in RA. *The Journal of Immunology*, 2010, 184: 3233–3241.

T helper-17 cells are a newly discovered CD4+ helper T cell subset that produce IL-17A (also known as IL-17). IL-17 binds to transmembrane receptors, which exist as IL-17 receptor A (IL-17RA) homodimers or IL-17RA/IL-17 receptor C (IL-17RC) heterodimers (1). IL-17 RA and RC are widely expressed in endothelial cells, B and T cells, fibroblasts, and monocytes (2, 3). TGF-β, IL-6, IL-1β, and IL-21 have been shown to drive the differentiation of Th-17 cells (4–6), although variation between humans and mice has been described previously (2, 6–8). In humans, IL-1β is the most effective inducer of Th-17 cell differentiation, although this process is enhanced by IL-6 and IL-23 (6).

IL-17 has been shown to have a profound effect in experimental models of arthritis. Local expression of IL-17 in mouse joints results in acute inflammation and cartilage proteoglycan depletion (9). The incidence and severity of collagen-induced arthritis was markedly attenuated in IL-17–deficient mice (10). IL-17 also plays an important role downstream of IL-1 signaling and in response to TLR4 ligands (11, 12). Spontaneous IL-1 receptor antagonist knockout mice develop a polyarthritis, which is completely suppressed when these mice are crossed with IL-17–deficient mice (13), suggesting that Th-17 cell differentiation may be the reason for the arthritis.

IL-17 is found in rheumatoid arthritis (RA) synovial tissue and fluid, and the percentage of Th-17 cells is significantly higher in RA synovial fluid compared with RA or normal peripheral blood (3, 14). IL-17 may play an important role in the pathogenesis of RA by inducing the production of proinflammatory cytokines and chemokines from RA synovial tissue fibroblasts including IL-6, CXCL8/IL-8, CCL2/MIP-3α, CXCL1/growth-related oncogene-α, and CXCL2/growth-related oncogene-β (15–17). IL-17 is also capable of activating macrophages to expression of IL-1, TNF-α, cyclooxygenase 2, PGE$_2$, and matrix metalloproteinase-9 (18–20). We recently demonstrated that IL-17 is directly chemotactic for monocytes (21), and others have shown that IL-17 activates neutrophil recruitment through the stimulation of chemokines such as CXCL1 in rats and CXCL8/IL-8 in humans (22, 23).

Angiogenesis, the development of new capillaries, is involved in leukocyte ingress into the synovium during the development and progression of RA (24, 25). However, the role of IL-17 in angiogenesis is undefined. There are contradictory results regarding the role of IL-17 on tumor development and angiogenesis. In mice, tumors transfected with IL-17 demonstrated markedly less growth compared with control tumors (26). Additionally, tumor growth and lung metastasis were increased in IL-17–deficient mice (27), suggesting that IL-17 inhibits tumor development and neovascularization. Others have shown that IL-17 increases blood vessel development in rat cornea and tumor vascularity in animal models, indicating that IL-17 may be important for angiogenesis (28). However, there are also data to suggest that IL-17 alone is unable to induce angiogenesis but can indirectly mediate human lung microvascular endothelial cell (HMVEC) growth by promoting the mitogenic activity of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (29, 30). Therefore, on the basis of the existing data, it is unclear whether IL-17 can promote angiogenesis in RA and whether IL-17 acts directly on endothelial cells through

binding to its receptors or indirectly by inducing proangiogenic factors from cells present in the RA synovium.

In the current study, we have therefore evaluated the role of IL-17 in HMVEC migration and tube formation as well as blood vessel development. Our results demonstrate that local expression of IL-17 increases vascularity in mouse ankle joints. Using a Matrigel plug assay, we demonstrate that IL-17 enhances blood vessel formation in vivo. Although HMVECs express both IL-17RA and RC, IL-17–induced HMVEC chemotaxis and tube formation are mediated by binding primarily to IL-17RC and activation of the PI3K pathway. We report that RA synovial fluid-mediated endothelial migration is significantly reduced by IL-17 and/or IL-17RC neutralization. Finally, we show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone. Hence, therapy directed against IL-17 may reduce leukocyte migration by inhibiting angiogenesis in RA.

## Materials and Methods

### Study protocol for local expression of IL-17 in mouse ankle joints

The animal studies were approved by the Northwestern University Institutional Review Board. Adenovirus (Ad) constructed as reported previously to contain an IL-17 payload was provided by J. K. Kolls (Louisiana State University Health Science Center, New Orleans, LA) (31). Four- to six-week-old C57BL/6 mice were injected intra-articularly with $10^7$ PFU Ad-IL-17 or Ad-CMV control. Ankle circumference and articular index score were determined on days 4 and 10 post–Ad-IL-17 injection and compared with the control group (data not shown). Ankles were harvested on day 10 post–Ad-IL-17 injection for histological studies. Levels of IL-17 were quantified by ELISA on days 4 and 10 from ankles treated with Ad-IL-17 or Ad-CMV control.

### Abs and immunohistochemistry

Mouse ankles were decalcified, formalin fixed and paraffin embedded, and sectioned in the pathology core facility of Northwestern University. Mouse ankles were immunoperoxidase stained using Vector Elite ABC Kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Slides were deparaffinized in xylene for 20 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by first incubating slides in boiling citrate buffer for 15 min, followed by type II trypsin digestion for 30 min at 37˚C. Endogenous peroxidase activity was blocked by incubation with 3% $H_2O_2$ for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories). Nonspecific binding of Abs to the tissues was blocked by pretreatment of tissues with diluted normal goat serum. Tissues were incubated with rabbit polyclonal Ab to von Willebrand factor (1/100 dilution; Serotec, Oxford, U.K.) or a rabbit IgG control Ab (Beckman Coulter, Fullerton, CA). Slides were counterstained with Harris hematoxylin and treated with lithium carbonate for bluing. Each slide was evaluated by a blinded observer (32–35) (M.V.V.). Tissue sections were assigned a vascular score representing the number of blood vessels in each section. A semiquantitative score of 1 represented a tissue with few blood vessels, whereas a score of 4 represented a highly vascularized tissue. Scored data were pooled, and the mean ± SEM was calculated in each data group ($n = 5$).

### Matrigel plug assay in vivo

To examine the effect of IL-17 on angiogenesis in vivo, we used a Matrigel plug assay. Four- to six-week-old C57BL/6 mice were injected s.c. in the dorsal area with 500 μl Matrigel. Matrigel plus PBS served as negative control, Matrigel containing bFGF (20 ng/ml) served as positive control, and experimental conditions included Matrigel with mouse recombinant IL-17 (2 μg). After 10 d, mice were sacrificed, Matrigel plugs were carefully dissected out, surrounding connective tissue was removed, and plugs were analyzed for vascularity by hemoglobin measurement or by histology. For hemoglobin measurement, plugs were weighed by placing them into 1 ml preweighed double-distilled $H_2O$ and then homogenized for 5–10 min on ice and spun. Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 to 1.1 g/dl (36, 37). Fifty microliters of supernatant or standard was added to a 96-well plate in duplicate, and 50 μl tetramethylbenzidine was added to each sample. The plate was allowed to develop at room temperature for 15–20 min with gentle shaking, and the reaction was terminated with 150 μl 2 N $H_2SO_4$ for 3–5 min. Absorbance was read with an ELISA

plate reader at 450 nm. To calculate hemoglobin concentrations, the values (grams per deciliter) were normalized to the weights of the plugs (grams) (36, 37). On day 10, IL-17 concentrations were quantified in Matrigel plugs harvested from the IL-17–treated group using ELISA.

For histology, plugs were embedded in paraffin and sectioned in the pathology core facility of Northwestern University. Histology slides from different groups were examined by H&E and Masson's trichrome staining (37, 38) and scored by a blinded observer (A.M.M.) based on a scale of 0–4, where 0 = no tubules, 1 = tubules only, 2 = tubules containing RBCs, 3 = tubules with multilayer wall, and 4 = tubules with multilayer walls with connective tissues surrounding them.

### HMVEC chemotaxis

To examine chemotaxis, HMVECs were incubated in endothelial basal medium (EBM) with 0% FBS and no growth factors for 2 h before use. HMVECs ($1.25 \times 10^4$ cells/25 μl EBM with 0.1% FBS) were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes (8-μm pore size; Nucleopore, Pleasant, CA) (37, 39). The chambers were inverted and incubated at 37˚C for 2 h, allowing endothelial cell attachment to the membrane. The chamber was reinverted, and PBS, positive control VEGF (10 ng/ml; R&D Systems, Minneapolis, MN), or IL-17 at varying concentrations from 0.001 to 100 ng/ml (R&D Systems) was added to the upper wells, and the chamber was further incubated for 2 h at 37˚C. The membranes were then removed, fixed, and stained with the Protocol HEMA 3 stain set (122-911; Fisher Scientific, Waltham, MA). Readings represent the number of cells migrating through the membrane (the average of three high-power ×40 fields/well, averaged for each triplicate of wells). To test specificity of IL-17–induced HMVEC migration, HMVEC chemotaxis was examined with heat-inactivated IL-17 (10 and 50 ng/ml incubated at 100˚C for 15 min) or IL-17 neutralized by anti–IL-17 Ab or IgG control (10 μg/ml for 1 h in 37˚C) (R&D Systems) (21). To examine for chemokinesis, a series of checkerboard experiments were performed by placing increasing concentrations of IL-17 (0, 0.01, 1, and 10 ng/ml) together with HMVECs in the bottom wells with different concentrations of IL-17 in the top wells. To determine which IL-17 receptors are important for IL-17 HMVEC migration, Abs to IL-17RA (R&D MAB177; 10 μg/ml), IL-17RC (R&D AF2269; 10 μg/ml), both Abs, or IgG control were added to HMVECs, incubated for 1 h at 37˚C, and further incubated for 2 h in the Boyden chamber with IL-17 (50 ng/ml). To define which signaling pathway(s) mediated IL-17–induced HMVEC chemotaxis, HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 2 h in the Boyden chamber with IL-17 (50 ng/ml). HMVEC chemotaxis induced by RA synovial fluids was examined following 1-h incubation (37˚C) of fluids with control IgG or neutralizing anti–IL-17 Ab (10 μg/ml). The fluids were diluted 1/20 before addition to the top wells (21). To examine whether IL-17 receptors are involved in RA synovial fluid-induced HMVEC chemotaxis, HMVECs were incubated with Abs to IL-17RA and RC (10 μg/ml) or both Abs, as well as IgG control (1 h at 37˚C), before adding the RA synovial fluid to the top wells (21). HMVEC chemotaxis was performed to examine the synergistic effect of IL-17 and/or VEGF in RA synovial fluid induced HMVEC migration. Chemotaxis induced by RA synovial fluids was examined following incubation of fluids (diluted 1/20) ($n = 8$ fluids) with control IgG, anti–IL-17, anti-VEGF, or both Abs (10 μg/ml) for 1 h prior to performing the assay.

### Quantification of IL-17RA and RC expression on HMVECs

Skin and lung HMVECs, HUVECs, and human embryonic kidney (HEK) 293 were cultured in EGM-2, EGM, and 10% DMEM, respectively. Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from all different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine IL-17RA and IL-17RC expression level as described previously (14, 21, 40). Relative gene expression was determined by the $\Delta\Delta C_t$ method, and results were expressed as fold increase above levels detected in HEK 293 cells.

### Characterization of IL-17 signaling pathways in HMVECs

HMVECs (passages 3–8) were grown to 80% confluence in EGM-2 MV bullet kit (Lonza, Walkersville, MD). Cells were then switched to EGM-MV (EBM plus SingleQuots of growth supplements) for 2 d and were incubated in EBM with 0% FBS for 2 h prior to treatment. Cells were then untreated or treated with IL-17 (50 ng/ml) for 15–75 min. Cell lysates were examined by Western blot analysis, as described previously (21, 40, 41). Blots were probed with phospho (p)-AKT, pERK, and pJNK (1/1000 dilution; Cell Signaling Technology, Beverly, MA) overnight and after stripping were probed with AKT, ERK, and JNK (1/3000 dilution; Cell Signaling Technology) overnight.

*HMVEC tube formation assay*

To perform the Matrigel tube formation assay, BD Matrigel Matrix (BD BioSciences, Bedford, MA; 50 μl) was polymerized for 30 min at 37°C in a 96-well plate. To determine which IL-17 receptors play a role in IL-17–induced HMVEC tube formation, trypsinized HMVECs were resuspended ($4 \times 10^5$ cells/ml) in EBM without growth factors with 2% FBS and incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC or IgG for 45 min at 37°C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml) was added to the wells, and the plate was incubated for 16 h at 37°C. To examine which signaling pathways contribute to IL-17–mediated HMVEC tube formation, cells were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 45 min at 37°C prior to adding to polymerized Matrigel. As above, IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C. Each condition was performed in triplicate, fibroblast growth factor (FGF) (20 ng/ml) was used as a positive control, and PBS was used as a negative control. Following incubation, culture medium was removed, and cells were washed with HBSS. Thereafter, tube formation was quantified using calcein-AM fluorescent dye (BD BioSciences) diluted with HBSS and DMSO, according to the manufacturer's instructions, for 30 min. Subsequently, the plates were washed with HBSS, and the number of branch points/tubes was quantified as described previously (36, 37).

*Statistical analysis*

The data were analyzed using two-tailed Student *t* tests for paired and unpaired samples. Values of $p < 0.05$ were considered significant.

## Results

*Local expression of IL-17 in mouse ankles induces joint inflammation and vascularity*

Local expression of IL-17 using an adenoviral vector ($10^7$ PFU) resulted in increased inflammation, synovial lining thickness, and bone erosion in the ankles of C57/BL6 mice, compared with Ad-CMV–infected controls ($10^7$ PFU) (data not shown). The Ad-IL-17–treated group demonstrated significantly greater ankle circumference (data not shown) on days 4 and 10 postinjection compared with the control group. Von Willebrand factor staining of ankles harvested from day 10 postinjection demonstrated that Ad-IL-17–treated mice have significantly higher endothelial staining compared with the control group (Fig. 1). The concentration of joint IL-17 in the IL-17–induced arthritis model was 1200 and 400 pg/mg compared with 47 and 31 pg/mg detected in the Ad-CMV
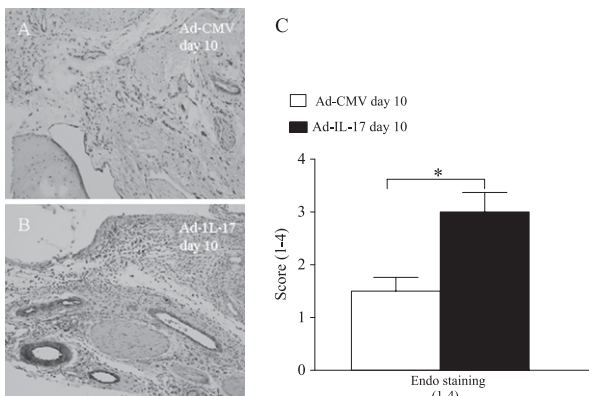


**FIGURE 1.** Local expression of IL-17 increases vascularity in mouse ankles. Ad-IL-17 or Ad-CMV control was injected intra-articularly into the ankle joints of 4–6-wk-old C57BL/6 mice. Ankles from day 10 post-Ad injection were harvested, paraffin embedded and decalcified. Ankles were stained with Von Willebrand factor for identifying endothelial cells (Endo) and scored on a 1–4 scale. Control ankles (*A*) had significantly lower endothelial staining compared with ankles locally expressing IL-17 (original magnification ×200) (*B*). *C*, Quantification of each of the parameters in the Ad-CMV control and Ad-IL-17 groups. Values demonstrate mean ± SE; *n* = 5. *$p < 0.05$.

control group on days 4 and 10 post-Ad injection. These results suggest that IL-17 may be important for angiogenesis in vivo.

*IL-17 induces angiogenesis in vivo in Matrigel plugs*

The role of IL-17 on angiogenesis in vivo was assessed by determining its effect on blood vessel formation in Matrigel plugs in mice by using hemoglobin quantification, as well as Masson's trichrome staining. The hemoglobin content of the IL-17–treated group was 10 times greater ($p < 0.05$) than the PBS control (Fig. 2*A*). Matrigel blood vessel formation was also examined histologically by using H&E (Fig. 2*C*, 2*E*, 2*G*) and Masson's trichrome staining (Fig. 2*D*, 2*F*, 2*H*). The histological analysis demonstrated that IL-17 markedly enhances ($p < 0.05$) blood vessel growth compared with the control group (Fig. 2*B*). The levels of IL-17 detected in Matrigel plugs harvested on day 10 were 198 ± 35 pg/ml, which is within the range detected in IL-17–induced arthritis model as well as in the RA synovial fluid (mean was 233 pg/ml). These results support the role of IL-17 in angiogenesis in vivo.

*IL-17 induces endothelial (HMVEC) migration*

Experiments were performed to determine whether IL-17 is directly chemotactic for endothelial cells. For this purpose, chemotaxis was performed in a Boyden chamber with varying concentrations of IL-17, as well as positive (VEGF; 10 ng/ml) and negative (PBS) controls. IL-17 was chemotactic for HMVECs at concentrations ranging from 0.01 ng/ml ($p < 0.05$) to 100 ng/ml ($p < 0.05$) ($n = 5$) (Fig. 3*A*). The mean concentration of IL-17 in the 30 RA synovial fluids analyzed was 233 ± 64 pg/ml (21), a concentration that was highly chemotactic for HMVEC migration. IL-17's endotoxin levels were quantified by using *Limulus* amebocyte cell lysate assay. At a concentration of IL-17 10-fold higher than that used in the HMVEC chemotaxis assay (500 ng/ml), the level of endotoxin was below the detection limit for this assay (<0.01 ng/ml LPS) (data not shown), suggesting that our results are specifically due to IL-17 and not endotoxin contamination. Supporting this interpretation, heat inactivation of IL-17 or incubation of IL-17 (50 ng/ml) with neutralizing Ab to IL-17 suppressed HMVEC migration (Fig. 3*B*).

We next determined the effect of IL-17 on chemokinesis. In the absence of IL-17 in the lower chamber, IL-17 in the upper chamber was chemotactic for HMVECs. When equivalent or higher concentrations of IL-17 were present in the lower compartment, no enhanced migration of HMVECs occurred (Fig. 3*C*). Taken together, our results suggest that IL-17 is chemotactic, not chemokinetic, for endothelial cells.

*IL-17 receptors are involved in IL-17–induced HMVEC chemotaxis and HMVEC tube formation*

Our results demonstrate that skin and lung HMVECs as well as HUVECs express significantly higher levels of IL-17RA and IL-17RC compared with HEK 293 cells (Fig. 4*A*, 4*B*). These experiments were performed to determine which IL-17 receptor is involved in HMVEC chemotaxis and tube formation. Although some reduction of HMVEC chemotaxis was noted with an anti–IL-17RA Ab, it was not significant. Inhibition of chemotaxis was significant when IL-17RC (40%) or both receptors (47%) were neutralized (Fig. 5*A*). IL-17 also induced HMVEC tube formation in Matrigel. Using concentrations of IL-17 ranging from 0.001 to 100 ng/ml, the optimal concentration of IL-17 to induce HMVEC tube formation was 50 ng/ml ($p < 0.05$) (data not shown). Consistent with the HMVEC chemotaxis data, neutralization of IL-17RC (30%) or both IL-17 receptors (45%) on HMVECs significantly decreased ($p < 0.05$) IL-17–mediated HMVEC tube formation (Fig. 5*B–H*). Blockade of IL-17RA alone was inefficient in this process. Collectively, our data suggest that IL-17RC plays a more important role

in IL-17–mediated HMVEC chemotaxis and tube formation compared with IL-17RA.

*IL-17 activates ERK, JNK, and PI3K pathways in HMVECs; however only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation*

To determine which signaling pathways in HMVECs are activated by IL-17, phosphorylation of the MAPK and AKT pathways was determined by Western blot analysis. Our data demonstrate that IL-17 phosphorylates ERK, JNK, and AKT1 as early as 15 min. Although the activation of ERK and JNK is gradually reduced at 75 and 65 min (Fig. 6B, 6C), respectively, AKT1 is still strongly phosphorylated at 75min (Fig. 6A). The p38 pathway was not activated by IL-17 in HMVECs (data not shown). To determine which signaling pathways mediate HMVEC migration, chemical inhibitors at concentrations of 1 and 5 μM were used, whereas 10 μM was toxic and resulted in cell death, as determined by trypan blue staining (data not shown). Inhibition of ERK and JNK was ineffective in suppressing IL-17–induced HMVEC chemotaxis, whereas inhibition of PI3K reduced ($p < 0.05$) chemotaxis starting at 1 μM (Fig. 6D). Similarly, although inhibition of PI3K (starting at 1 μM) reduced IL-17–mediated tube formation by 30–40% ($p < 0.05$) (Fig. 7), suppression of ERK and JNK had no effect on this process. These results suggest that IL-17–induced HMVEC chemotaxis and tube formation are mediated through the PI3K/AKT1 pathway.

*IL-17 and its receptor are involved in RA synovial fluid-mediated HMVEC chemotaxis*

Next, we asked whether the IL-17 identified in human RA synovial fluid is chemotactic for HMVECs. In these experiments, we demonstrated that human RA synovial fluid is chemotactic for HMVECs, similar to positive control VEGF (Fig. 8A). Furthermore, human RA synovial fluid immunodepleted with anti–IL-17 significantly reduced HMVEC chemotaxis ($p < 0.05$), compared with control IgG-treated fluids. Neutralization of IL-17RA and RC on HMVECs showed that only IL-17RC was effective in suppressing human RA synovial fluid-mediated HMVEC migration (Fig. 8B). These results suggest that IL-17 and its receptor IL-17RC may play an important role in angiogenesis in RA. Furthermore, we demonstrate that immunodepletion of IL-17 and VEGF in RA synovial fluids does not have an additive or synergistic effect in reduction of HMVEC migration beyond the effect noted with neutralization of one factor alone (Fig. 8C), suggesting that both IL-17 and VEGF may be mediating HMVEC migration through the same signaling pathway.

## Discussion

In this study, we show that IL-17 increases vascularity in experimental arthritis and induces blood vessel development in Matrigel plugs in vivo. Therefore, studies were performed to determine whether IL-17 might directly mediate angiogenesis and whether IL-17 contributes to neovascularization in RA. Our data demonstrate that IL-17 induces HMVEC chemotaxis at concentrations present in human RA synovial fluid. This effect is directly mediated by IL-17, because heat inactivation and neutralizing Abs to IL-17 and/or IL-17 receptors abrogate IL-17–induced HMVEC chemotaxis. We further demonstrate that IL-17–induced HMVEC chemotaxis and tube formation are mediated primarily through ligation to IL-17RC on HMVECs and activation of PI3K. We show that human RA synovial fluid-mediated HMVEC chemotaxis is markedly reduced by neutralization of IL-17 in the synovial fluids or blocking of IL-17RC on HMVECs. Last, we demonstrate that IL-17 and VEGF-immunodepleted RA synovial fluid does not reduce HMVEC chemotaxis any further than neutralization of each factor by itself.

In this paper, we confirm the results of others demonstrating that the local expression of IL-17 in mouse ankle joints induces arthritis (9). Histological analysis of mice receiving intra-articular injections of IL-17 demonstrated that IL-17 plays an important role in joint neutrophil migration (42). We demonstrate that IL-17–induced arthritis is associated with increased vascularity. Others have shown that IL-17 can promote tumor growth by upregulating proangiogenic factors such as VEGF and matrix metalloproteinase-9 from tumor cells, suggesting that IL-17 is indirectly associated with angiogenesis (43). We have also shown that forced ectopic expression of IL-17 induces expression of proangiogenic CXC (ELR[+]) chemokines in mouse ankles (S. Pickens and S. Shahrara, unpublished data).

On the basis of our results from the IL-17–induced arthritis model, we hypothesized that IL-17 may be important for angiogenesis in RA. Because there is some evidence demonstrating that

**FIGURE 2.** IL-17 enhances blood vessel growth in Matrigel plugs in vivo. *A*, IL-17 (2 μg) induced angiogenesis in the Matrigel plugs to a significantly greater degree compared with PBS control. Matrigel containing bFGF (20 ng/ml) served as positive control. The values represent the concentration of hemoglobin (grams per deciliter)/plug weight (grams) ± SE, with n = 10. *B*, The histology was quantified on a score of 0–4 scale in Matrigel plugs that contained PBS, IL-17, or bFGF using H&E and Masson's trichrome staining. A representative assay shows H&E (*C, E, G*) and Masson's trichrome (*D, F, H*) staining of blood vessels in paraffin sections of Matrigel plugs containing PBS (*C, D*), IL-17 (*E, F*), and FGF (*G, H*) that was histologically scored in *B* (original magnification ×200). Values demonstrate mean ± SE, with n = 5. *$p < 0.05$.
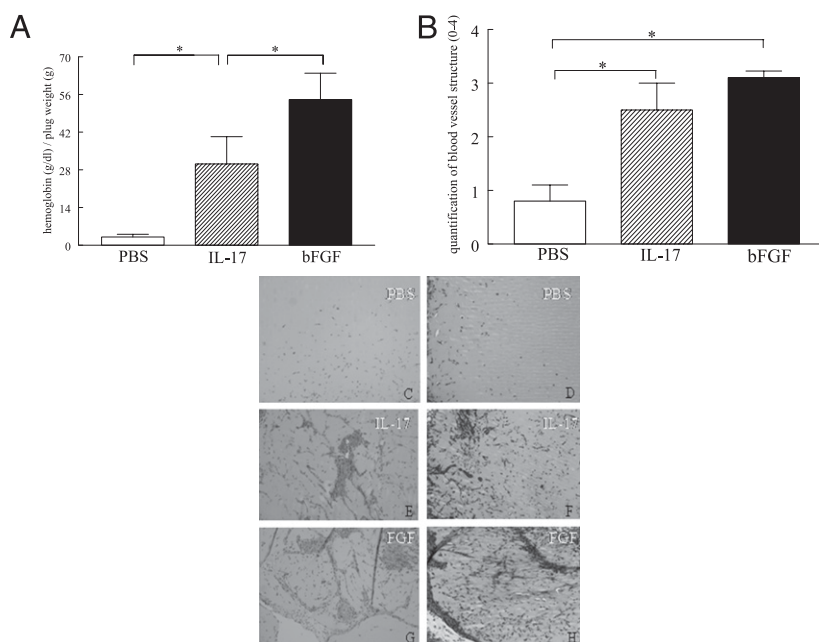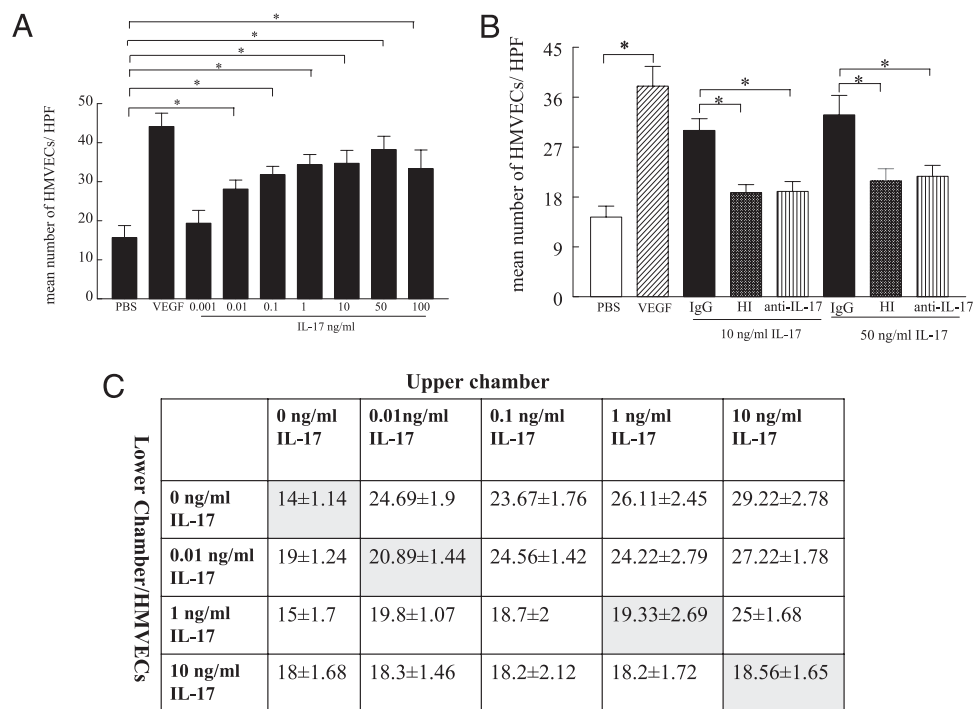
**FIGURE 3.** IL-17 induces HMVEC migration and not chemokinesis. *A*, Dose-response curve of IL-17–induced HMVEC chemotaxis. IL-17 HMVEC chemotaxis was performed in a Boyden chemotaxis chamber with varying concentrations of IL-17. Values demonstrate mean ± SE from five different experiments. *B*, IL-17–induced HMVEC chemotaxis was suppressed by heat inactivating IL-17 (both 10 and 50 ng/ml incubated in 100°C for 15 min) or neutralization of IL-17 (10 and 50 ng/ml) by anti–IL-17 Ab but not by IgG control (10 μg/ml 1 h in 37°C) for 2 h. Values are the mean ± SE from three different experiments. *p < 0.05. *C*, A series of checkerboard experiments was performed by placing increasing doses of IL-17 together with HMVECs in the lower well in addition to placing different concentrations of IL-17 in the top well of the chemotaxis chamber. The experiment was read at 2 h. Results are representative of three different experiments.

IL-17 alone is unable to induce angiogenesis but can indirectly promote HMVEC chemotaxis by producing proangiogenic factors (29, 30) from RA synovial tissue fibroblasts, we investigated the role of IL-17 on HMVEC migration and tube formation. Our results demonstrate that IL-17 induces HMVEC chemotaxis at concentrations available in the human RA joint, which is mostly



**FIGURE 4.** HMVECs express IL-17RA (*A*) and RC (*B*). Skin and lung HMVECs, HUVECs, and HEK 293 were cultured and real-time RT-PCR was performed to determine IL-17RA and IL-17RC expression level. The relative gene expression levels were normalized by GAPDH and determined by the $\Delta\Delta C_t$ method, and results were expressed as fold increase above levels detected in HEK 293 cells. Values are the mean ± SE, *n* = 3–6. *p < 0.05.

due to its ligation to IL-17RC. Although IL-17RC plays a major role in IL-17–mediated HMVEC chemotaxis and tube formation, neutralization of both receptors is more effective in this process compared with IL-17RC alone. Like monocytes, HMVECs express both IL-17RA and IL-17RC (21). However, in contrast to HMVEC chemotaxis, IL-17–mediated monocyte migration is induced through both IL-17RA and RC (21). Interestingly, a novel IL-17 receptor-like protein has been identified in HUVECs that interacts with FGF1 and inhibits activation of the ERK pathway and production of FGF, indicating that various IL-17 receptors may modulate angiogenesis differently (44).

Next, experiments were performed to investigate signaling pathways that were associated with IL-17–induced HMVEC chemotaxis and tube formation. Inhibition of the IL-17–activated pathways in HMVECs demonstrated that only activation of PI3K significantly reduces IL-17–mediated chemotaxis and tube formation, and suppression of ERK and JNK pathways was ineffective in this process. Consistently, CCL2/MCP-1–mediated endothelial chemotaxis is through activation of PI3K, as well as the ERK pathway (45). Furthermore, others have shown that PI3K signaling plays an important role in regulation of VEGF production as well as VEGF-mediated endothelial migration (46, 47), suggesting that PI3K is involved in the mediation of angiogenesis by various proinflammatory factors. A recently published article shows that increased expression of IL-17 in IFNγ$^{-/-}$ mice mediates tumor growth and angiogenesis through STAT3 phosphorylation (43). In contrast to IL-17, which promotes angiogenesis, IL-17F inhibits HUVEC tube formation, indicating that different IL-17 isoforms may modulate angiogenesis differently (48).

A recent publication demonstrated that tumor growth in s.c. and lung metastases are enhanced in IL-17$^{-/-}$ mice compared with the wild-type controls, suggesting that IL-17 may suppress tumor
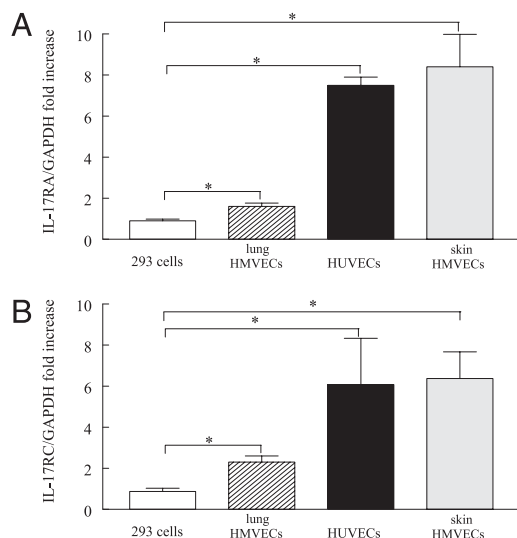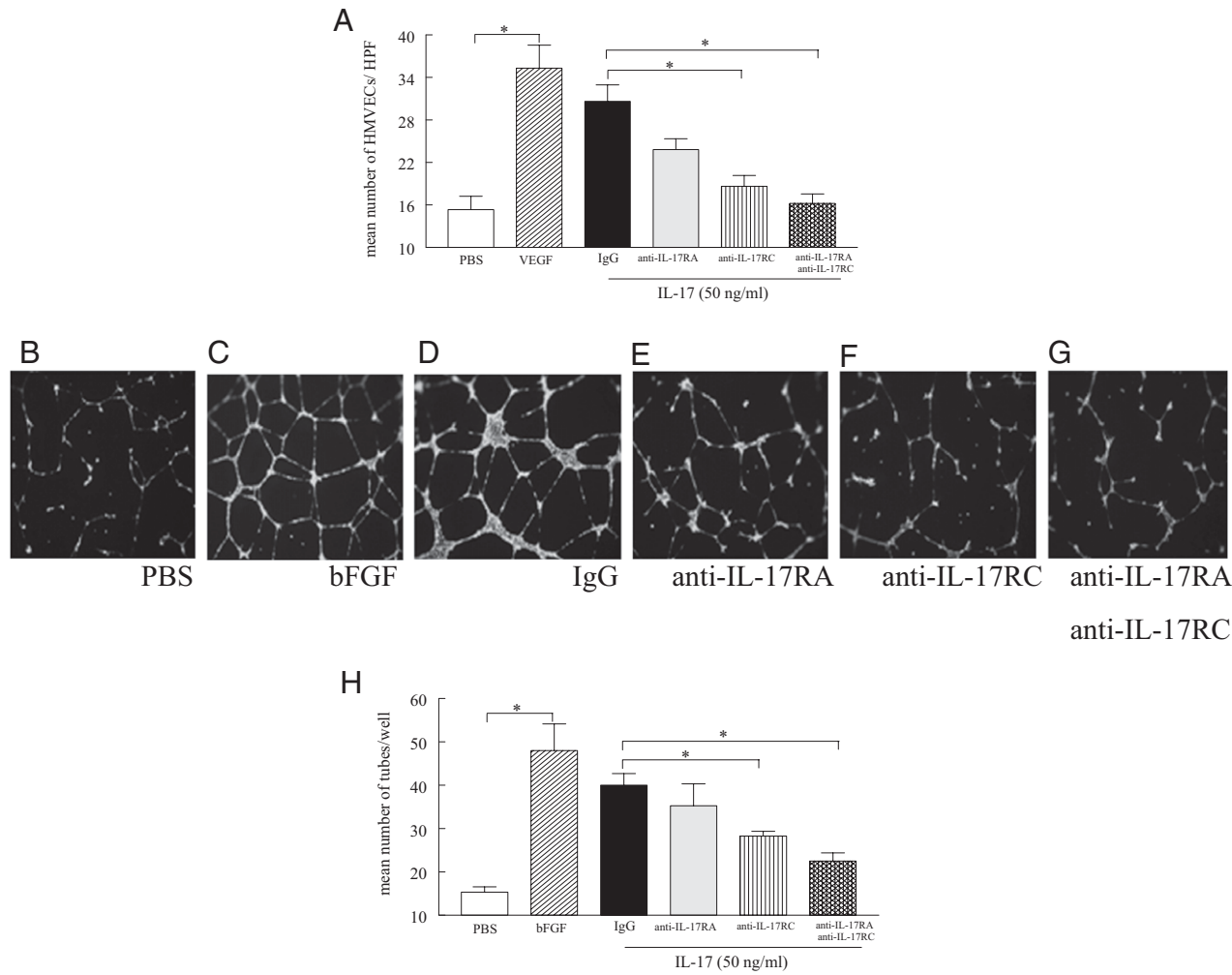
**FIGURE 5.** IL-17–mediated HMVEC chemotaxis and tube formation are regulated through both IL-17RA and IL-17RC. *A*, HMVECs were incubated with mouse anti-human IL-17RA and IL-17 RC Abs (10 μg/ml) or control IgG (10 μg/ml) for 1 h. Thereafter, HMVEC chemotaxis was performed in response to IL-17 (50 ng/ml) for 2 h. PBS was used as a negative control and VEGF (60 nM) as a positive control. HMVECs were incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC, or IgG for 45 min at 37°C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml), placed in the wells, and the plate was incubated for 16 h at 37°C (in triplicate). Photomicrographs (original magnification ×100) taken of representative wells treated with PBS (*B*), FGF (20 ng/ml) (*C*), IL-17 (50 ng/ml) plus IgG (*D*), IL-17 (50 ng/ml) plus anti–IL-17RA (10 μg/ml) (*E*), IL-17 (50 ng/ml) plus anti–IL-17RC (10 μg/ml) (*F*), and IL-17 (50 ng/ml) plus anti–IL-17RA and RC (10 μg/ml) (*G*) in which IL-17–induced tube formation is significantly reduced by the neutralization of IL-17RC or both receptors ($p < 0.05$). *H*, Data presented demonstrates mean number of branch points/tubes in each treatment group. Values are the mean $\pm$ SE, $n = 3$. $*p < 0.05$.

development (27). Conversely, others have shown that IL-17 markedly increases neovascularization in rat cornea (28) and vascularization in tumors (49), indicating that IL-17 may promote angiogenesis. However, there is also evidence demonstrating that IL-17 induces production of proangiogenic factors including NO, hepatocyte growth factor, CXCL1/KC, CXCL2/MIP-2, PGE$_1$,
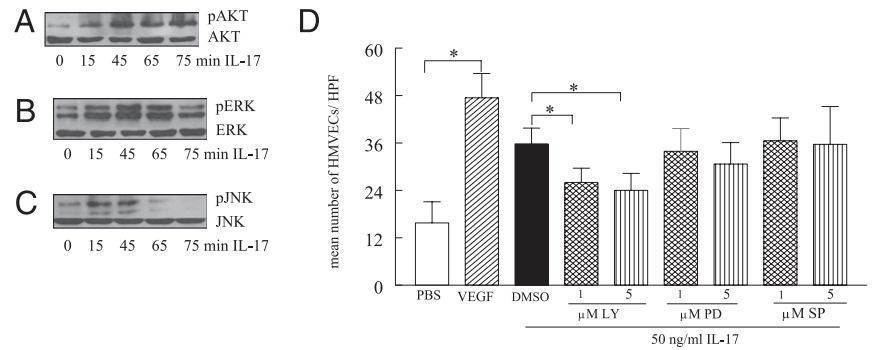


**FIGURE 6.** IL-17–induced HMVEC migration is suppressed by PI3K inhibition. To determine the mechanism of IL-17 in HMVECs, cells were stimulated with IL-17 (50 ng/ml) for 0–75 min, and the cell lysates were probed for p-AKT (*A*), pERK (*B*), or pJNK (*C*). These results are representative of three experiments. *D*, To determine signaling pathways associated with IL-17 HMVEC migration, cells were treated with the identified chemical inhibitors for PI3K (LY294002; 1 and 5 μM) or ERK (PD98059; 1 and 5 μM) as well as JNK (SP600125; 1, 5 μM) 2 h in the Boyden chamber. Only inhibition of PI3K downregulated IL-17–induced HMVEC migration. Values demonstrate mean $\pm$ SE of three experiments in triplicate. $*p < 0.05$.

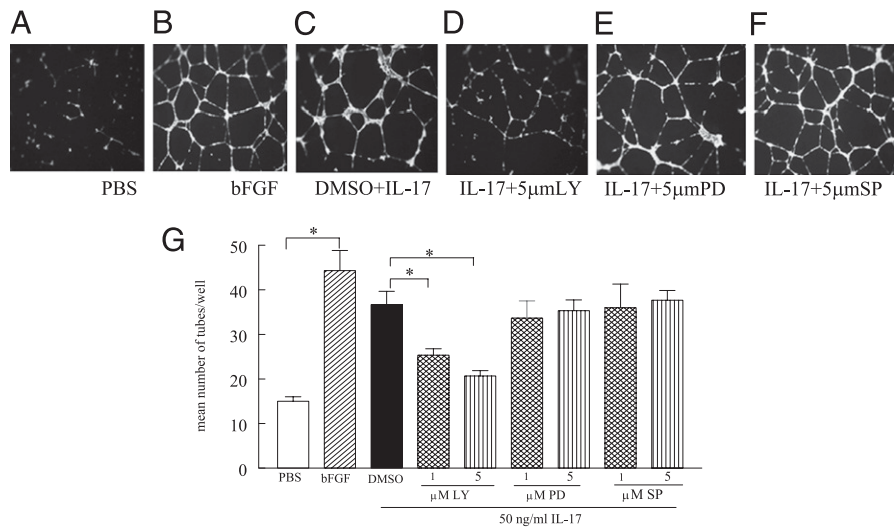**FIGURE 7.** IL-17–mediated tube formation is reduced by inhibition of PI3K. HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 45 min at 37°C prior adding to polymerized Matrigel. IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C (in triplicate). Photomicrographs (original magnification ×100) taken of representative wells treated with PBS (*A*), FGF (20 ng/ml) (*B*), IL-17 (50 ng/ml) plus DMSO (*C*), IL-17 (50 ng/ml) plus LY294002 (5 μM) (*D*), IL-17 (50 ng/ml) plus PD98059 (5 μM) (*E*), and IL-17 (50 ng/ml) plus SP600125 (5 μM) (*F*) in which IL-17–induced tube formation is significantly reduced by the inhibition of PI3K/AKT1 pathway (*p* < 0.05). *G* demonstrates mean number of tubes per well where LY294002 (1 and 5 μM; PI3K/AKT1 inhibitor) significantly reduces the number of branch points induced by IL-17 activation in Matrigel tube formation assay, whereas ERK (PD98059; 1 and 5 μM) and JNK inhibitors (SP600125; 1 and 5 μM) were ineffective. Values are the mean ± SE, *n* = 3. ∗*p* < 0.05.

PGE$_2$, and VEGF from RA synovial fibroblasts, and the production of some of these factors is further enhanced by TNF-α (50). Therefore, we investigated the contribution of IL-17 to human RA synovial fluid-mediated HMVEC chemotaxis. Neutralization of IL-17 in RA synovial fluid partially reduced RA synovial fluid-mediated HMVEC chemotaxis. RA synovial fluid-mediated HMVEC chemotaxis was mediated through IL-17RC, confirming the importance of this receptor in IL-17–mediated angiogenesis. Interestingly, angiogenic factors present in human RA synovial fluid are mostly produced by RA synovial tissue fibroblasts (VEGF, bFGF, VCAM1, IL-6, and ELR$^+$ CXC chemokines) or macrophages (TNF-α, IL-8, and IL-1β) (25). IL-17 is the only
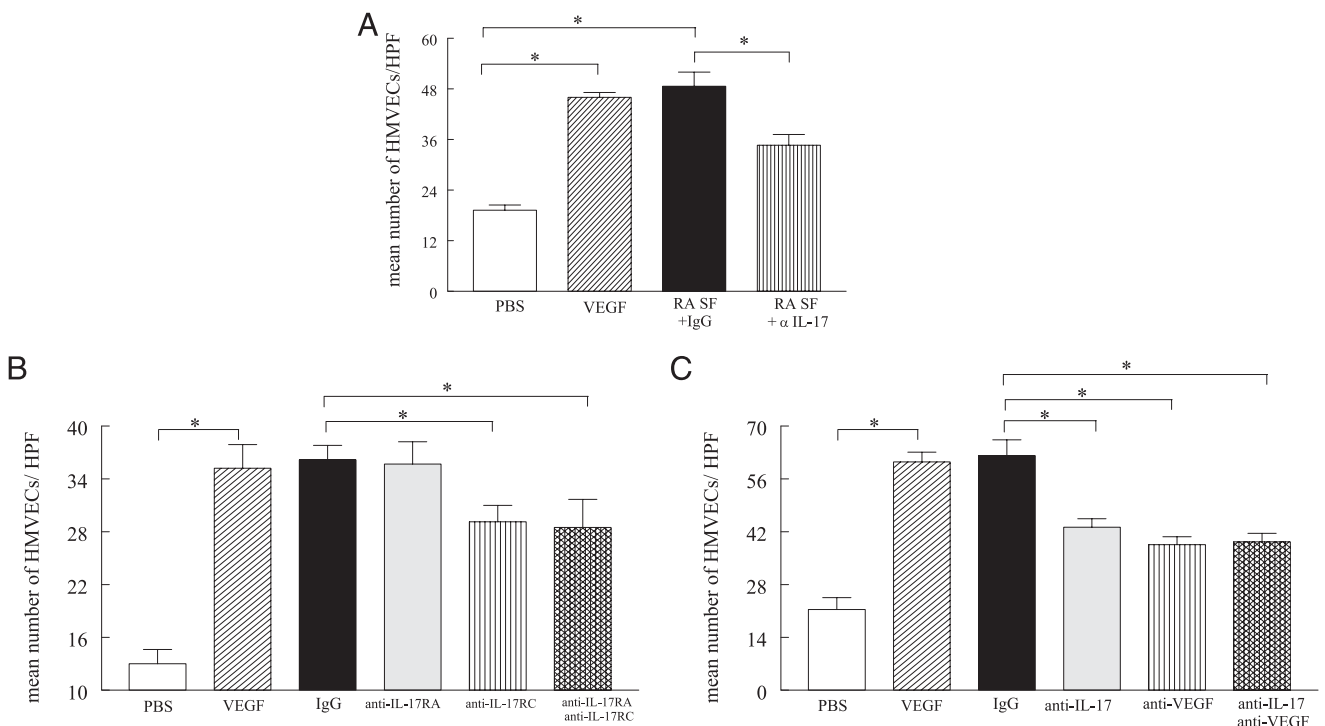


**FIGURE 8.** RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 through ligation to IL-17RC, and IL-17 does not synergize with VEGF in RA synovial fluid-induced HMVEC migration. *A*, Anti–IL-17 (10 μg/ml) or control IgG was added to RA synovial fluids from eight patients (1/20 dilution) (1 h at 37°C) prior to performing HMVEC chemotaxis in response to human RA synovial fluids. *B*, HMVECs were incubated with Abs to IL-17 RA and RC (10 μg/ml), as well as isotype control for 1 h prior to performing HMVEC chemotaxis in response to eight human RA synovial fluids. *C*, RA synovial fluids from eight patients (1/20 dilution) were incubated with Abs to IL-17 (10 μg/ml), VEGF (10 μg/ml), or both as well as isotype control or PBS or VEGF for 1 h prior to performing HMVEC chemotaxis in response to RA synovial fluids. The values represent the mean ± SE. ∗*p* < 0.05.

lymphokine that contributes to human RA synovial fluid-mediated angiogenesis, suggesting that T cells may also be important in this process. The data presented in this study, together with our previously reported evidence demonstrating that IL-17 is important for monocyte migration (21) in RA synovial fluids, highlight the importance of IL-17 in RA pathogenesis.

Interestingly, our results show that neutralization of IL-17 and VEGF do not synergize in reducing RA synovial fluid-induced HMVEC migration beyond the effect detected with one factor alone. As shown with IL-17, VEGF-induced HMVEC chemotaxis is mediated through PI3K (46, 51). Therefore, the lack of synergy between IL-17 and VEGF in inducing HMVEC chemotaxis may be due to that both mediators are using the same signaling pathway or that as shown previously HMVEC cell migration is within a bell shaped curve (37, 52); therefore, the synergistic effect could not be detected.

In conclusion, endothelial migration and tube formation induced by IL-17 were mediated through activation of the PI3K pathway and ligation to both IL-17 receptors. However, neutralization of only IL-17 or IL-17RC significantly downregulated human RA synovial fluid-mediated endothelial migration, suggesting that IL-17 plays an important role in RA angiogenesis.

## Disclosures

The authors have no financial conflicts of interest.

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# Local Expression of Interleukin-27 Ameliorates Collagen-Induced Arthritis

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*Objective*. To determine the mechanism of action of interleukin-27 (IL-27) against rheumatoid arthritis (RA).

*Methods*. Adenovirus containing IL-27 transcript was constructed and was locally delivered into the ankles of mice with collagen-induced arthritis (CIA). Progression of arthritis was determined in treated and untreated mice by measuring ankle circumference and through histologic analysis. IL-17 and its downstream targets as well as cytokines promoting Th17 cell differentiation were quantified by enzyme-linked immunosorbent assay in CIA mouse ankles locally expressing adenoviral IL-27 as well as in control-treated mouse ankles. Ankles from both treatment groups were immunostained for neutrophil and monocyte migration (macrophages in the tissue). Finally, vascularization was quantified by histology and by determining ankle hemoglobin levels.

*Results*. Ectopic expression of IL-27 in CIA mice ameliorated inflammation, lining hypertrophy, and bone erosion as compared with control-treated CIA mice. Serum and joint levels of IL-17 were significantly reduced in the IL-27–treated group compared with the control-treated group. Two of the main cytokines that induce Th17 cell differentiation and IL-17 downstream target molecules were greatly down-regulated in CIA mouse ankles receiving forced expression of IL-27. The control mice had higher levels of vascularization and monocyte trafficking than did mice ectopically expressing IL-27.

*Conclusion*. Our results suggest that increased levels of IL-27 relieve arthritis in CIA mouse ankles. This amelioration of arthritis involves a reduction in CIA mouse serum and joint levels of IL-17 and results in decreased IL-17–mediated monocyte recruitment and angiogenesis. Hence, the use of IL-27 may be a strategy for treatment of patients with RA.

Interleukin-17 (IL-17) is found in rheumatoid arthritis (RA) synovial fluid and in the T cell–rich areas of RA synovial tissue (1,2). Th17 cells, which are derived from RA synovial tissue, are significantly increased in RA synovial fluid compared with RA or normal peripheral blood (3). Our recent studies have shown that IL-17 mediates angiogenesis in RA synovial fluid through ligation to IL-17 receptor C (4). IL-17 can also contribute to the pathogenesis of RA by inducing monocyte migration into the inflamed synovial tissue (5,6).

IL-17 plays a profound role in the pathogenesis of experimental arthritis. Collagen-induced arthritis (CIA) is markedly reduced in IL-17$^{-/-}$ mice (7), and treatment of the experimental CIA model with anti–IL-17 antibody decreases the severity of inflammation and bone destruction (8). Further, local expression of IL-17 increases inflammation and synovial lining thickness (9), which we have shown to be associated with increased vascularity and monocyte recruitment (3,6).

IL-27 is a heterodimeric cytokine produced by macrophages and dendritic cells; it belongs to the IL-12 cytokine family, which includes IL-23 and IL-35 (10). IL-27 is composed of 2 subunits, Epstein-Barr virus–

induced gene 3 and p28, whose transcriptions are regulated independently. As such, dissociation of the expression of the 2 subunits may occur (11). Dendritic cells produce IL-27 when stimulated by pathogen-associated molecular patterns through Toll-like receptors (TLRs) (12). We have shown that macrophages from RA synovial fluid have significantly higher levels of IL-27 production compared with control cells; however, both groups of cells produced similar levels of IL-27 in the presence of TLR-2 ligation (3). Consistent with this, others have shown that IL-27 is expressed in RA synovium (13).

IL-27 mediates its proinflammatory effect by modulating the initial step of Th1 cell differentiation through the induction of IL-12 receptor $\beta2$ (IL-12R$\beta$2) expression, which can lead to interferon-$\gamma$ (IFN$\gamma$) production (14,15). Concordant with these results, IL-27R$^{-/-}$ mice demonstrated reduced inflammation in the proteoglycan-induced arthritis model by downregulating IFN$\gamma$–producing T cells (16). In contrast, IL-27 can also suppress inflammation by inhibiting murine Th17 cell differentiation mediated by IL-6 and transforming growth factor $\beta$ (TGF$\beta$). It was shown that the absence of IL-27 increased the severity of experimental autoimmune encephalomyelitis (EAE) by promoting T cell proliferation and Th17 cell differentiation (17,18). Further, EAE in IL-27R$\alpha^{-/-}$ mice was ameliorated by using antibody against IL-17 (17). The suppressive effect of IL-27 was distinct from that of IFN$\gamma$, since EAE induced in double knockouts of IFN$\gamma$ and IL-27R$\alpha$ was more severe than in each single knockout alone. It was further shown that IL-27 is a potent suppressor of Th17 cell development in a STAT-1–dependent and IFN$\gamma$-independent way (17,18). Others have shown that the antiinflammatory properties of IL-27 may also be due to induction of IL-10 by CD4+ cells through a STAT-1– and STAT-3–dependent pathway (19).

Experiments were performed to examine the mechanism by which IL-27 affects the pathogenesis of CIA. Our results demonstrated that 2 of the cytokines promoting Th17 cell differentiation, as well as downstream targets of IL-17 in macrophages and fibroblasts, were significantly reduced in ankles adenovirally expressing IL-27 compared with ankles injected with control phosphate buffered saline (PBS). Ectopic expression of IL-27 in the ankles down-regulated CIA vascularization and monocyte migration into synovial tissue compared with the control group. Using RA memory T cells, we demonstrated that while IL-27 treatment significantly reduced the percentage of Th17 cells, it had no effect on Th1 cells. These results suggest that inhibition

of Th17 cell polarization through IL-27 may be a useful RA treatment.

## MATERIALS AND METHODS

**Construction of AdIL-27.** Mouse IL-27 complementary DNA (cDNA) was obtained from p3xFLAG-IL-27 plasmid described previously by Matsui et al (20), and AdIL-27 was constructed by Welgen. Briefly, IL-27 cDNA was cloned into pCR-TOPO vector (Invitrogen) by polymerase chain reaction. Thereafter, IL-27 cDNA was released with *Bgl* II and ligated to pENT-CMV predigested with the same enzyme, and the positive clones were screened by digestion with *Bam* HI and sequenced. The pENT-IL-27 cDNA was treated with LR Clonase II enzyme (Invitrogen) and ligated to a pAdREP plasmid that contains the remaining adenovirus genome. The recombination products were transformed into *Escherichia coli* cells, and after overnight incubation, the positive clones were selected, and cosmid DNA was purified. The purified cosmid DNA (2 $\mu$g) was digested with *Pac* I and then transfected into 293 cells with Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). The 293 cells were grown in Dulbecco's modified Eagle's medium. The adenovirus plaques were seen 7 days after transfection. The concentration of the AdIL-27 was $3 \times 10^{10}$ plaque-forming units (PFU) as determined by plaque assay. The control adenovirus (AdControl) used in this study was an empty pENT-CMV shuttle vector with no insert (adenovirus purchased from Welgen).

**Transfection of AdIL-27 in 293 cells and detection of AdIL-27 in mouse ankles.** We cultured 293 cells in a 6-well plate to 50–75% confluence. The next day, cells were infected at 0, 5, 10, or 25 multiplicities of infection (MOI) of AdIL-27. Following a 48-hour incubation, conditioned medium and cells were collected. The conditioned medium was concentrated using 30-kd columns (VWR Scientific Products); AdIL-27 was detected in the conditioned medium and cell lysates by probing for FLAG (1:3,000 dilution), and equal loading was determined by actin (1:3,000 dilution) or by staining with Coomassie blue. Mice were injected intraarticularly (IA) with $10^5$, $10^6$, or $10^7$ PFU of AdIL-27 (injected into both ankles) or with control PBS, and ankles were harvested after 5 days. Ankles were then homogenized in a 50-ml conical centrifuge tube containing 1 ml of Complete Mini-protease inhibitor cocktail (Roche Molecular Biochemicals) homogenization buffer. Ankle homogenization was completed on ice using a motorized homogenizer, followed by 30 seconds of sonication. Homogenates were centrifuged at 2,000$g$ for 10 minutes and filtered through a 0.45-$\mu$m pore size Millipore filter (21–23). AdIL-27 expression was examined in ankle homogenates through Western blot probing of FLAG (1:3,000 dilution), and equal loading was examined by actin (1:3,000 dilution).

**Study protocol for CIA and AdIL-27 treatment.** DBA/1J mice (age 7–8 weeks) were immunized with collagen on days 0 and 21. Bovine type II collagen (2 mg/ml; Chondrex) was emulsified in an equal volume of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* H37Ra; Difco or Chondrex). The DBA/1J mice were immunized subcutaneously in the tail with 100 $\mu$l of emulsion. On day 21, mice were injected intradermally with 100 $\mu$l of type II collagen (2 mg/ml) emulsified in an equal volume of Freund's incomplete adjuvant. AdIL-27 ($10^7$ PFU, n = 15) or AdControl ($10^7$ PFU, n = 15) was injected IA on day 23 after CIA induction. Mice were

killed on day 42; ankles were harvested for protein and messenger RNA extraction, as well as for histologic studies, and serum was saved for laboratory tests.

**Clinical assessments.** Ankle circumferences were determined by measurement of 2 perpendicular diameters, including the laterolateral diameter and the anteroposterior diameter, using calipers (Lange Caliper; Cambridge Scientific). Circumference was determined using the following formula: circumference $= 2\pi\sqrt{(a^2 + b^2/2)}$, where a and b represent the diameters. Ankle circumference evaluations were performed on days 21, 23, 26, 28, 30, 33, 35, 36, and 41.

**Flow cytometry.** RA peripheral blood mononuclear cells were isolated by Histopaque gradient centrifugation, and memory CD4+ T cells were isolated with a negative selection kit (StemCell Technologies) according to the manufacturer's instructions. RA memory CD4+ T cells were cultured and treated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1 μg/ml), with or without IL-27 treatment (100 ng/ml) for 48 hours. The cells were supplemented with brefeldin A (10 μg/ml) 18 hours prior to performing flow cytometry. Cells were then stained with anti-CD4 (RPA-T4; BD PharMingen), anti–IL-17 (eBio64DEC17; eBioscience), anti-IFNγ (4S.B3; BD PharMingen), or isotype control antibodies. Th17
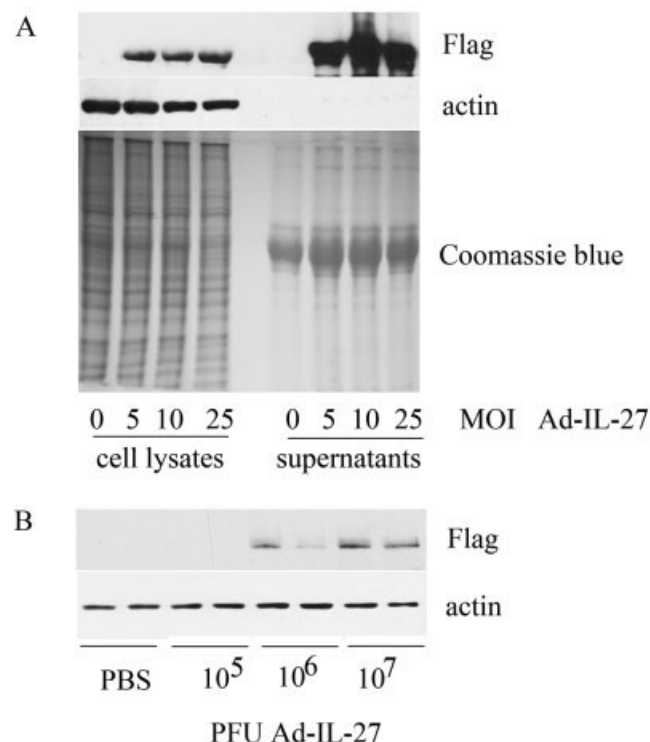


**Figure 1.** Western blot analysis of AdIL-27 construct. **A,** Western blot analysis using anti-FLAG, antiactin, or Coomassie blue protein staining of lysates and conditioned medium obtained from 293 cells transfected with 0, 5, 10, or 25 multiplicities of infection (MOI) of AdIL-27. **B,** Western blot analysis using anti-FLAG or antiactin antibody on homogenates of mouse ankles injected with phosphate buffered saline (PBS) control or with $10^5$, $10^6$, or $10^7$ plaque-forming units (PFU) of AdIL-27.
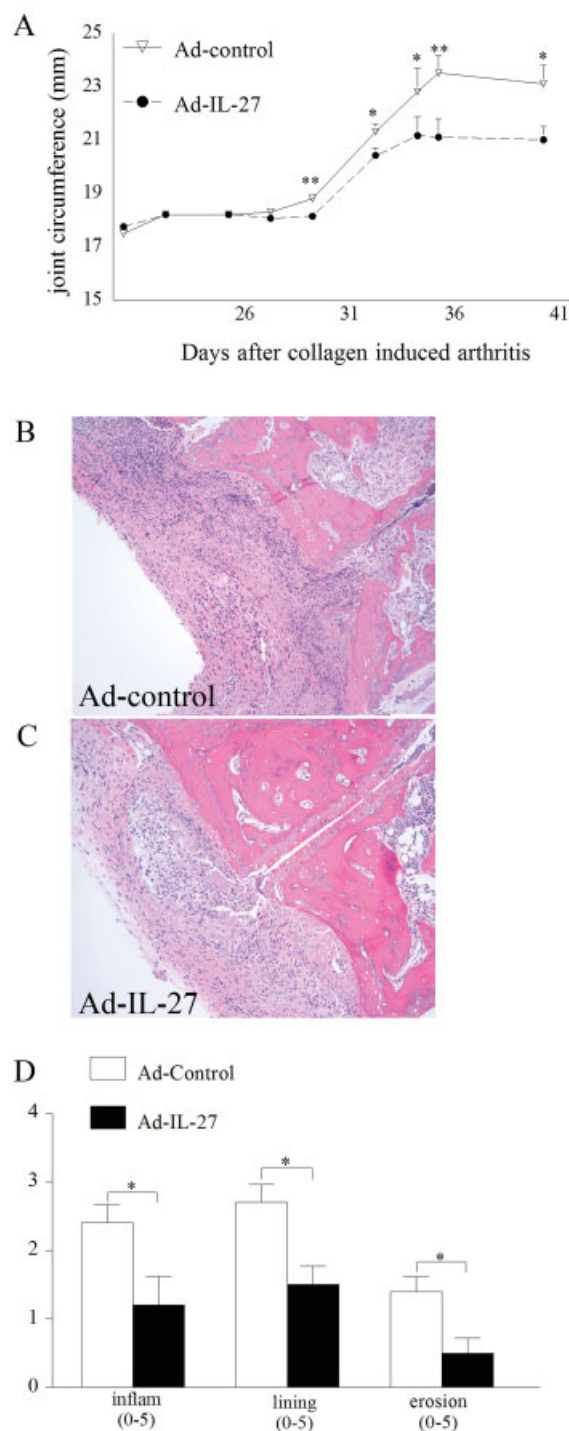


**Figure 2.** Local expression of interleukin-27 (IL-27) ameliorates the pathology of collagen-induced arthritis (CIA). **A,** Changes in joint circumference. $* = P < 0.05$; $** = P < 0.01$ versus AdIL-27–treated mice. **B** and **C,** Hematoxylin and eosin staining of CIA mouse ankles injected with control adenovirus (AdControl) (**B**) or AdIL-27 (**C**). Original magnification $\times$ 200. **D,** Effect of local expression of IL-27 on inflammation, lining thickness, and bone erosion. $* = P < 0.05$. Values in **A** and **D** are the mean $\pm$ SEM (n = 10 mice).
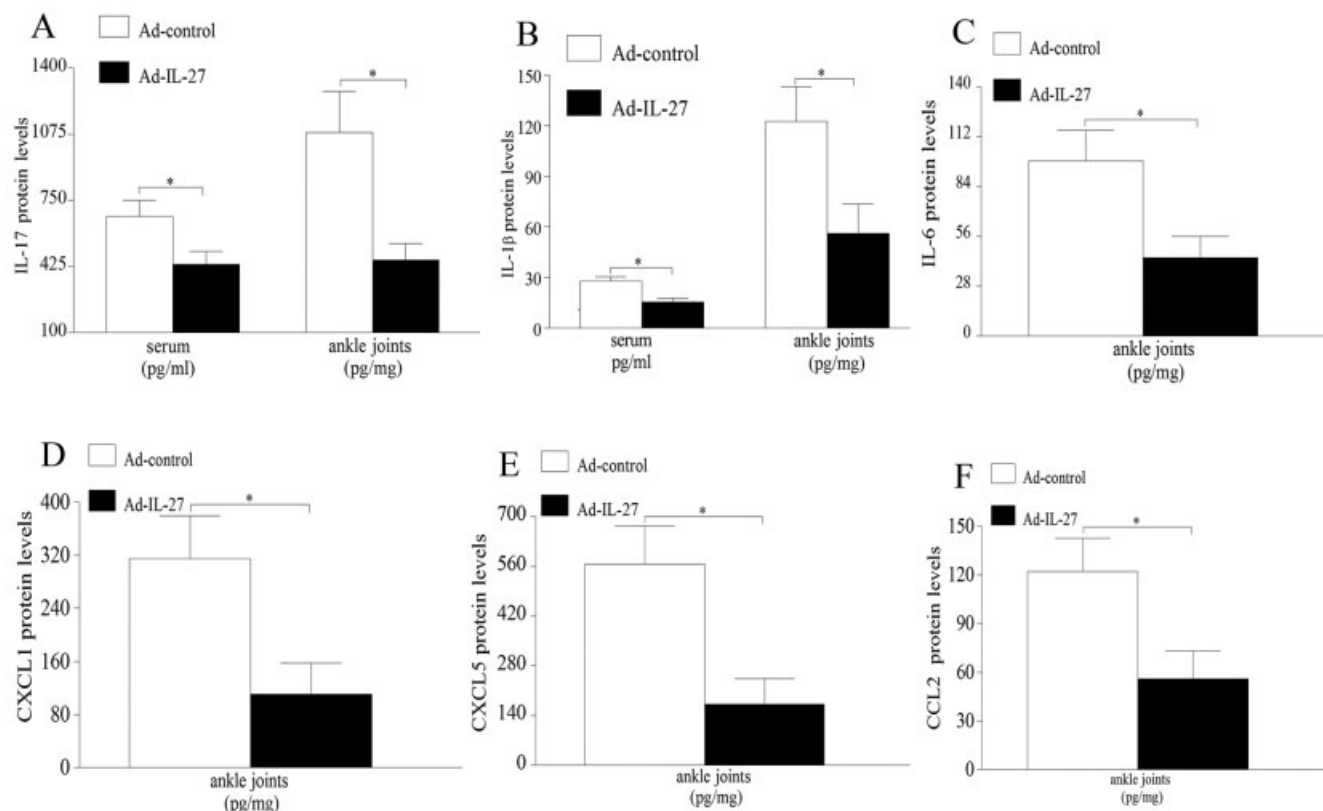
**Figure 3.** Forced expression of IL-27 reduces levels of IL-17 and proinflammatory factors in the joint. **A** and **B,** Levels of expression of IL-17 (**A**) or IL-1$\beta$ (**B**) in sera and ankle homogenates from CIA mice treated with AdControl or AdIL-27 were determined by enzyme-linked immunosorbent assay (ELISA). **C–F,** Levels of expression of IL-6 (**C**), CXCL1 (**D**), CXCL5 (**E**), or CCL2 (**F**) in ankle homogenates from both treatment groups were quantified by ELISA. Values are the mean $\pm$ SEM (n = 9–10 mice). $*$ = $P < 0.05$. See Figure 2 for other definitions.

or Th1 cells were identified as CD4+IL-17+ or CD4+IFN$\gamma$+, respectively.

**Antibodies and immunohistochemistry.** Mouse ankles were decalcified, fixed in formalin, embedded in paraffin, and were sectioned in the pathology core facility of Northwestern University. Inflammation, synovial lining thickness, and bone erosion (scored on a 0–5 scale) were determined by a blinded observer (AMM) viewing hematoxylin and eosin–stained sections. Mouse ankles were stained with immunoperoxidase using Vector Elite ABC Kits (Vector), with diaminobenzidine (Vector) as a chromogen, at the pathology core facility of Northwestern University.

Briefly, slides were deparaffinized in xylene for 15 minutes at room temperature, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (Dako) for 5 minutes at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% $H_2O_2$ for 5 minutes. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako). Nonspecific binding of antibodies to the tissues was blocked by pretreatment of tissues with Protein block (Dako). Tissues were incubated with GR1 (1:200 dilution; Novus Biologicals), MAC 387 (1:200 dilution; Serotec together with animal research kit [ARK; Dako]), von Willebrand factor (1:1,000 dilution; Dako), or control IgG

antibody (Beckman Coulter). Slides were counterstained with Mayer's hematoxylin and treated with lithium carbonate for bluing. Neutrophil and macrophage staining were scored on a 0–5 scale. Vascularity was quantified as number of blood vessels per 5 random high-power fields at 10$\times$ magnification (24). The data were pooled, and the mean $\pm$ SEM was calculated in each data group. Each slide was evaluated by a blinded observer (AMM) (22,23,25,26).

**Quantification of proinflammatory factors.** Mouse ankle and/or serum IL-17, IL-1$\beta$, IL-6, tumor necrosis factor $\alpha$ (TNF$\alpha$), CXCL1, CXCL5, CCL20, and CCL2 were quantified by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (R&D Systems). The sensitivity of the ELISAs performed to quantify mouse IL-17, IL-1$\beta$, IL-6, CXCL1, and CXCL5 was 7.8 pg/ml, whereas for TNF$\alpha$ and CCL20 it was 15.6 pg/ml, and for CCL2 it was 1.95 pg/ml. The expression level of each factor was normalized to the ankle protein concentration and reported as pg/mg, and serum levels are reported as pg/ml.

**Quantification of hemoglobin in mouse ankles.** Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 gm/dl to 1.1 gm/dl (4,27,28). Fifty microliters of homogenized mouse ankles or standard was added to a 96-well plate in duplicate, and 50 $\mu$l of tetra-methylbenzidine was added to each sample. The plate was
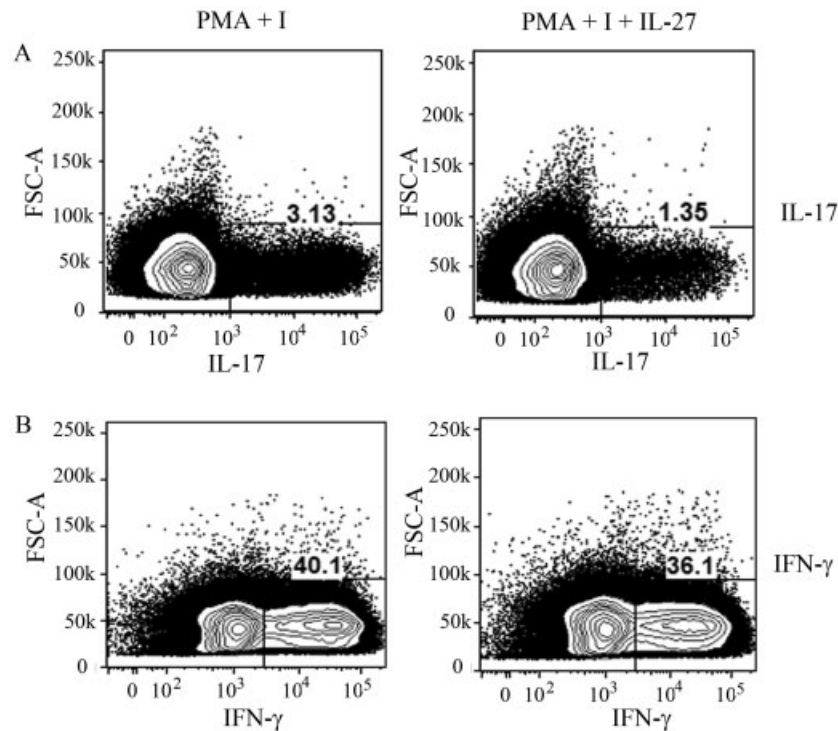
**Figure 4.** Interleukin-27 (IL-27) treatment reduces the percentage of Th17 cells but not of Th1 cells in rheumatoid arthritis (RA) peripheral blood. **A** and **B,** Percentages of CD4+IL-17+ cells (**A**) or CD4+ interferon-$\gamma$ (IFN$\gamma$)–positive cells (**B**) were quantified in RA peripheral blood memory CD4+ T cells treated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (I; 1 $\mu$g/ml) or with PMA, ionomycin, and IL-27 (100 ng/ml) for 48 hours. The cells were supplemented with brefeldin A (10 $\mu$g/ml) 18 hours before flow cytometry was performed. Results demonstrate that IL-27 treatment reduces Th17 cells without affecting Th1 cells. FSC = forward scatter.

allowed to develop at room temperature for 15–20 minutes with gentle shaking, and the reaction was terminated with 150 $\mu$l of $2N$ $H_2SO_4$ for 3–5 minutes. Absorbance was read with an ELISA plate reader at 450 nm. To calculate hemoglobin concentrations in the mouse ankles, the values (gm/dl) were normalized to the weights of the ankles (mg/ml) (4,27,28).

**Statistical analysis.** The data were analyzed using Student's 2-tailed $t$-tests for paired and unpaired samples. $P$ values less than 0.05 were considered significant.

## RESULTS

**Expression of AdIL-27 in transfected 293 cells and in mouse ankles.** To verify that AdIL-27 was capable of expressing IL-27, 293 cells were transfected with 0, 5, 10, and 25 MOI of AdIL-27. Following a 48-hour incubation, protein expression was determined by Western blotting of both cell lysates and conditioned medium. Using anti-FLAG antibody we were able to detect AdIL-27 construct at 5, 10, and 25 MOI from both 293 cell lysates and conditioned medium (Figure 1A).

To validate the expression of IL-27 in vivo, mouse ankles were bilaterally injected with $10^5$, $10^6$, or $10^7$ PFU of AdIL-27 or PBS control. After 5 days, ankles were homogenized and AdIL-27 construct was detected employing anti-FLAG antibody in Western blotting analysis. AdIL-27 construct was detectable only in ankles injected IA with $10^6$ or $10^7$ PFU AdIL-27, and not in ankles injected IA with $10^5$ PFU AdIL-27 or PBS control (Figure 1B). Since both mouse ankles injected with $10^7$ PFU AdIL-27 strongly expressed IL-27 compared with only 1 mouse ankle injected with $10^6$ PFU AdIL-27, the dose of $10^7$ PFU was selected for performing these experiments.

**Effect of local IL-27 expression in CIA.** To determine the effect of IL-27 administration into the arthritic joint, AdIL-27 or AdControl ($10^7$ PFU each) was injected IA into DBA/1J mouse ankles 23 days after CIA induction. In the AdControl-injected mice, disease activity determined by ankle circumference began on
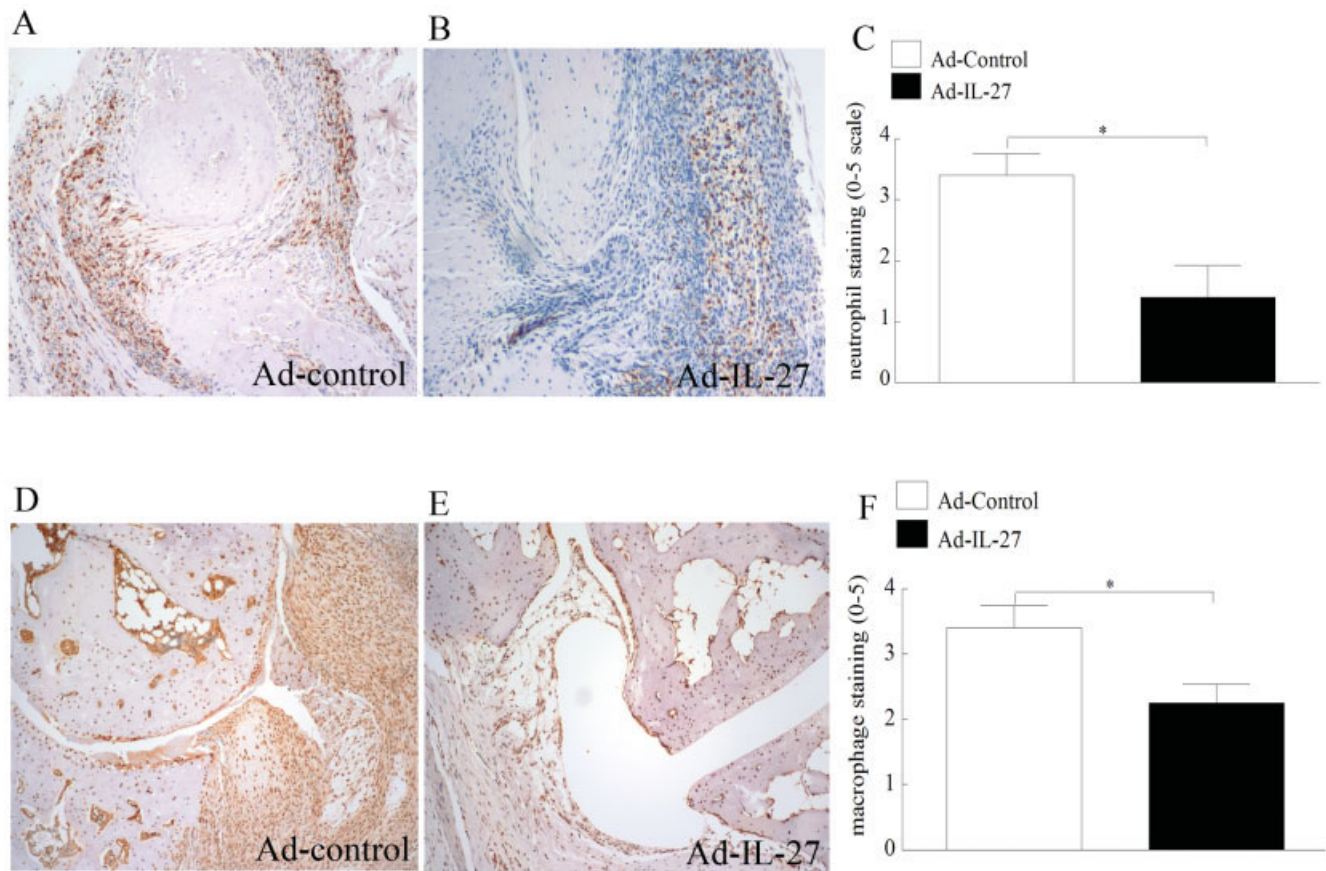
**Figure 5.** Local expression of IL-27 down-regulates CIA-mediated neutrophil and monocyte ingression. Synovial tissue from AdControl- or AdIL-27–injected CIA mouse ankles harvested on day 42 was immunostained with GR1 (neutrophil marker) (**A** and **B**) or MAC 387 (macrophage marker) (**D** and **E**). Original magnification × 200. Neutrophil staining (**C**) and macrophage staining (**F**) of CIA mouse ankles harvested on day 42 were assessed and quantified. Values are the mean ± SEM (n = 10 mice). ∗ = $P < 0.05$. See Figure 2 for definitions.

day 30 and progressed through day 36, plateauing thereafter until the termination of the experiments on day 42 (Figure 2A). Mice treated with AdIL-27 demonstrated significantly reduced joint circumference compared with control animals ($P < 0.05$ and $P < 0.01$). Next, histologic examination of the joints was performed to determine the effect of treatment on inflammation and joint destruction. Histologic analysis of ankles obtained on day 42 confirmed that mice treated with AdIL-27 had significantly less inflammation (50% decrease), synovial lining thickness (45% decrease), and bone erosion (65% decrease) than did control mice (Figures 2B–D). These results suggest that local expression of IL-27 can reduce CIA joint inflammation, synovial lining thickness, and bone destruction.

**Effect of AdIL-27 treatment on expression of proinflammatory factors in CIA mouse ankles.** IL-27 is known to suppress inflammation by inhibiting Th17 cell differentiation; therefore, IL-17 expression levels were determined in serum and ankles of CIA mice receiving

AdIL-27 or AdControl. Our results demonstrate that IL-17 expression was significantly lowered in sera and ankle homogenates (by 35% and 55%, respectively) from AdIL-27–treated animals compared with those from control animals (Figure 3A). Interestingly, we demonstrated that 2 of the cytokines that drive Th17 cell differentiation, namely, IL-1β and IL-6, were significantly reduced (by 55%) in mouse ankles, and levels of IL-1β were also decreased (by 45%) in sera from CIA mice locally expressing IL-27 compared with sera from mice receiving control treatment (Figures 3B and C). We have shown that CXCL1, CXCL5, and CCL2 are neutrophil and monocyte chemokines that are induced by IL-17 in RA synovial tissue fibroblasts and macrophages as well as in a model of IL-17–induced arthritis (ref. 6 and Pickens SR, Chamberlain ND, Shahrara S: unpublished observations). We found that ectopic expression of IL-27 significantly decreased joint levels of CXCL1 (by 65%), CXCL5 (by 70%), and CCL2 (by 55%) compared with AdControl treatment in CIA (Fig-
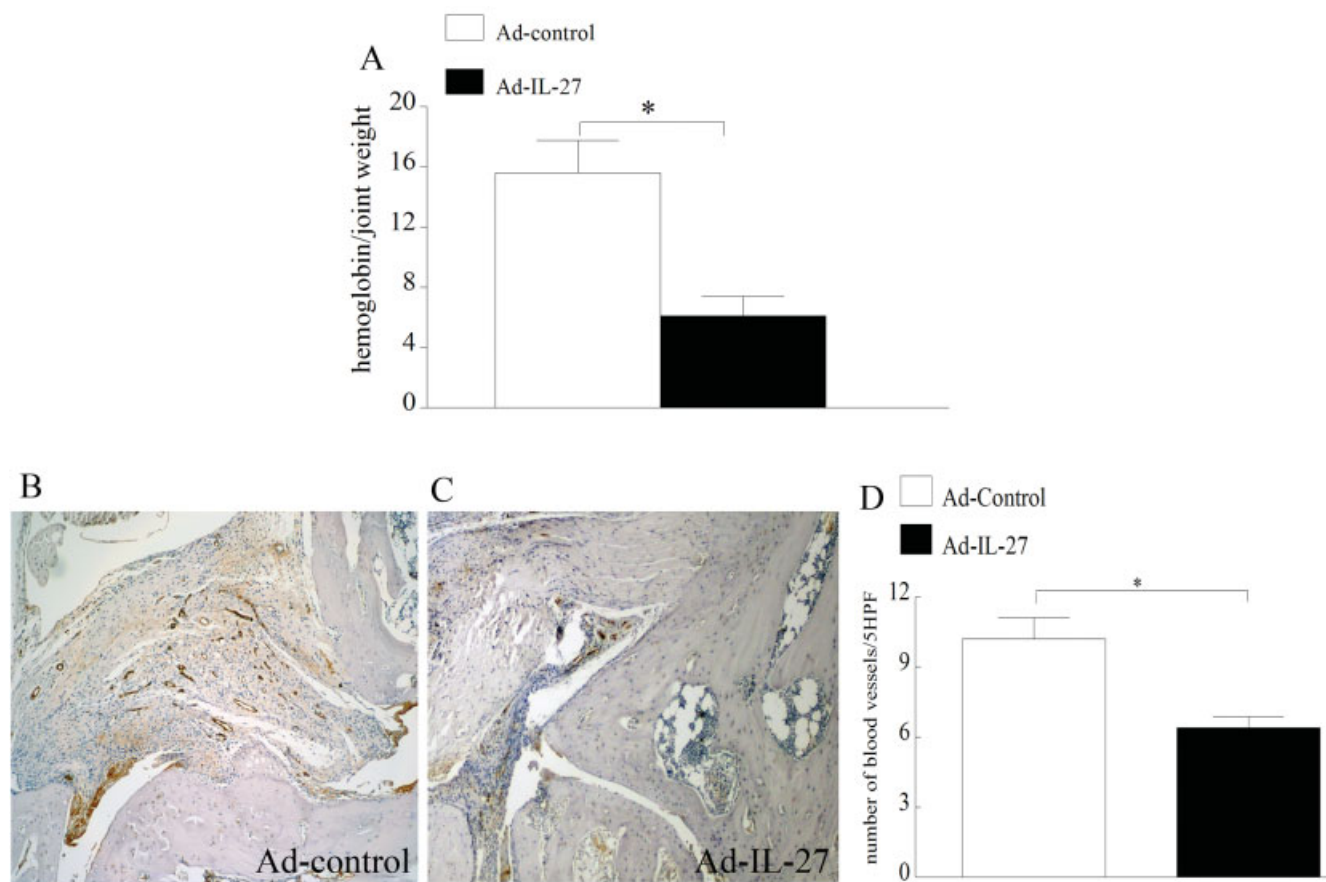
**Figure 6.** Reduced vascularization detected in CIA mouse ankles locally expressing IL-27. **A,** Hemoglobin levels in CIA mouse ankles harvested from different treatment groups on day 42 were quantified. Results are shown as hemoglobin (gm/dl)/joint weight (mg/ml). **B** and **C,** Synovial tissue from AdControl-injected (**B**) or AdIL-27–injected (**C**) CIA mouse ankles harvested on day 42 was immunostained with von Willebrand factor (endothelial marker). Original magnification × 200. **D,** Vascularization was quantified as the number of blood vessels per 5 random high-power fields (hpf) at 10× magnification in each CIA mouse ankle harvested on day 42. Values in **A** and **D** are the mean ± SEM (n = 10 mice). ∗ = $P < 0.05$. See Figure 2 for other definitions.

ures 3D–F). These results suggest that local expression of IL-27 could suppress Th17 cell polarization as well as IL-17 downstream target genes.

**Treatment with IL-27 significantly reduces RA Th17 cells without affecting Th1 cells.** Since splenocytes and T cells are difficult to transfect due to low expression of adenovirus receptor, in order to demonstrate that IL-27 can directly reduce CD4+IL-17+ cells, RA peripheral blood memory T cells were isolated by negative selection and were treated with PMA and ionomycin with or without IL-27. Results from these experiments demonstrate that while IL-27 treatment significantly reduced Th17 cells from 3% to 1%, it had no effect on the Th1 cell population (Figures 4A and B). Consistently, when IL-17 levels in conditioned medium were quantified by ELISA (after 48 and 72 hours), cells treated with IL-27 had lower secretion of IL-17 compared with cells in the control treatment group (data not shown).

**Effect of AdIL-27 treatment on leukocyte recruitment into CIA mouse joints.** We have shown thus far that local expression of IL-27 in CIA mouse ankles significantly reduced inflammation as well as Th17 cell polarizing cytokines and IL-17–induced downstream factors. We next examined the effect of AdIL-27 on leukocyte recruitment into inflamed CIA mouse ankle joints. Consistent with the clinical data, local expression of IL-27 greatly suppressed the ingress of neutrophil (by 60%) (Figures 5A–C) and monocytes (by 35%) (Figures 5D–F) into CIA mouse joints as compared with control mouse joints. Our results suggest that reduction of joint IL-17 levels can down-regulate trafficking of neutrophils and monocytes into CIA mouse ankles.

**Effect of AdIL-27 treatment on vascularization in CIA.** Since angiogenesis is critical for leukocyte ingress, the effect of local IL-27 expression on blood vessel formation in CIA mice was studied. Vascularization in CIA mice was quantified by measuring ankle hemoglo-

bin levels and blood vessel staining. AdIL-27–treated CIA mice had markedly lower hemoglobin levels compared with control mice (Figure 6A). Consistently, there were 40% fewer blood vessels in CIA mouse ankles that locally expressed IL-27 than in control mouse ankles (Figures 6B–D). Our results suggest that IL-27 treatment inhibits IL-17–mediated angiogenesis in CIA.

## DISCUSSION

In this study, we showed that local expression of IL-27 in CIA mouse ankles ameliorates joint inflammation and bone destruction. We further demonstrated that IL-27 modulates arthritis through reducing 2 important Th17 cell polarizing cytokines as well as IL-17–activated factors in the CIA mouse joint. In RA peripheral blood, IL-27 treatment directly reduced the percentage of Th17 cells; however, Th1 cells were unaffected. Consequently, local expression of IL-27 in CIA mouse ankles suppresses IL-17–mediated neutrophil and monocyte trafficking as well as vascularization. These results suggest that IL-27 can inhibit IL-17–induced acute (neutrophil migration) and chronic (monocyte recruitment) inflammation by affecting leukocyte ingress, controlled in part by the reduction in angiogenesis.

Early neutralization of IL-17 using an IL-17 receptor IgG Fc fusion protein in CIA suppresses the onset of the disease (29). Treatment of CIA after disease onset using anti–IL-17 antibody decreases the severity of inflammation and bone destruction in CIA (8). These studies demonstrated that IL-17 plays an important role in the initiation and progression of CIA. Hence, we investigated whether inhibition of Th17 cell differentiation could reduce joint inflammation in CIA. The effect of local expression of IL-27 on Th17 cells was observed both systemically and in the ankle joints, since IL-17 levels in the sera and ankle homogenates were markedly decreased compared with those in the control group. Experiments were performed in RA peripheral blood in order to demonstrate that IL-27 treatment could directly inhibit Th17 cell differentiation and that reduction in IL-17 levels was distinct from IFNγ–mediated Th17 cell suppression. Consistent with previous findings (17,18), we showed that Th17 cell differentiation was suppressed 3-fold while Th1 cell polarization was unaffected by IL-27 treatment in RA peripheral blood.

We found that local expression of IL-27 could alleviate clinical signs of CIA. Consistent with this, histologic analysis demonstrated reduced inflammation, synovial lining thickness, and bone erosion, which may be due to suppressed joint levels of IL-17. It has been shown that IL-17 is involved in bone degradation through elevating the expression of RANKL in CIA mouse ankles (30) as well as through synergizing with TNFα and IL-6 in this process (31,32).

TGFβ, IL-6, IL-1β, and IL-21 drive the differentiation of Th17 cells (33–35). However, some variation between humans and mice has been described. Levels of IL-1β and IL-6, but not TNFα, were markedly reduced in CIA mouse ankles locally expressing IL-27 compared with control mouse ankles. Others have shown that in IL-1Ra$^{-/-}$ mice, elevated levels of IL-1β are responsible for an increase in the number of Th17 cells (36). In CIA, IL-6 is essential for Th17 cell differentiation, since anti–IL-6R antibody markedly suppresses induction of Th17 cells and arthritis development (37). Consistent with our data, a previous study demonstrated that systemic administration of recombinant IL-27 could reduce serum levels of IL-6 (13). Interestingly, in CIA mouse synoviocytes, neutralization of IL-1β and IL-6 significantly reduces IL-17–mediated expression of TLRs 2, 4, and 9 (38). The results from our laboratory and others suggest that IL-1β and IL-6 are 2 of the cytokines that play an important role in Th17 cell differentiation in CIA. Therefore, IL-27 can suppress polarization of Th17 cells by modulating joint levels of IL-1β and IL-6.

Our unpublished studies demonstrated that IL-17 can induce CXCL1 expression from RA synovial tissue fibroblasts, macrophages, and human microvascular endothelial cells. CXCL5 is also produced from IL-17–activated RA synovial tissue fibroblasts and macrophages (Pickens SR, Chamberlain ND, Shahrara S: unpublished observations). Previous studies have shown that neutrophil migration mediated by IA injection of IL-17 is dependent on CXCL1 and CXCL5, suggesting that both neutrophil chemokines are produced by cells in the ankle joints and play an essential role in IL-17–mediated neutrophil ingress (39). Neutrophil chemotaxis caused by conditioned media from IL-17–stimulated gastric epithelial cells was inhibited by neutralizing antibodies to IL-8, suggesting that in human cells IL-8 is responsible for IL-17–induced neutrophil trafficking (40). Similar to IL-8, CXCL1 and CXCL5 bind to CXCR2 and therefore may induce neutrophil migration through activation of the same pathway. Collectively, the data suggest that reduction of IL-17 by AdIL-27 suppresses neutrophil migration through modulating CXCL1 and CXCL5 in CIA mouse ankle joints.

We have shown that IL-17 plays an important role in monocyte migration in RA, since neutralization of IL-17 in RA synovial fluid or its receptors on monocytes significantly reduces monocyte migration mediated by RA synovial fluid (5). Further, IL-17 promotes mono-

cyte migration through activation of p38 MAPK (5). We also found that IL-17 activates CCL2 production by macrophages and RA synovial tissue fibroblasts and in experimental arthritis models (6). In addition to the direct effect of IL-17 on monocyte chemotaxis, we demonstrated that IL-17–mediated monocyte recruitment into the peritoneal cavity was due in part to CCL2 production (6). Despite the ability of IL-17 to induce the production of other monocyte chemokines, such as CCL20, from cells present in the synovial lining (6), forced expression of IL-27 in CIA mouse ankles did not affect the expression levels of this chemokine. Based on our previous studies, inhibition of monocyte recruitment into CIA mouse ankles locally expressing IL-27 may be directly due to reduction of IL-17 levels or indirectly due to lower expression of IL-17–induced CCL2, or perhaps both mechanisms are essential for this process.

Angiogenesis is an early and critical event in the pathogenesis of RA, which is triggered by the inflammatory process mediated by cytokines, chemokines, and hypoxia (41). Previous studies demonstrated that angiogenesis is essential for CIA progression (42). In the current study, we showed that local expression of IL-27 significantly reduced synovial vascularity in CIA mice compared with that in control animals. This effect may be due to down-regulation of joint levels of IL-17 in ankles with forced IL-27 expression. We previously observed that IL-17, in concentrations present in the RA joint, induces endothelial migration through the phosphatidylinositol 3-kinase/Akt-1 pathway (4). Further, we have demonstrated that IL-17 is angiogenic, as determined by its ability to promote blood vessel growth in Matrigel plugs in vivo (4). However, reduced levels of the proangiogenic chemokines CXCL1 and CXCL5 may also be responsible for the decreased vascularity in the AdIL-27 treatment group compared with that in the control group (43). Given that angiogenesis promotes the ingress of leukocytes, reduction in new blood vessel formation can affect neutrophil and monocyte trafficking.

In summary, local expression of IL-27 in CIA results in reduced disease severity quantified by joint swelling, synovial lining thickness, bone erosion, and leukocyte migration. In CIA, AdIL-27 treatment leads to reduced IL-1$\beta$ and IL-6 production, resulting in a depressed Th17 cell response characterized by decreased joint levels of IL-17. This leads to decreased synovial production of the neutrophil and monocyte chemokines CXCL1, CXCL5, and CCL2, ultimately resulting in fewer infiltrating leukocytes and less blood vessel formation.

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ORIGINAL PAPER

# Anti-CXCL5 therapy ameliorates IL-17-induced arthritis by decreasing joint vascularization

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**Abstract** IL-17-induced joint inflammation is associated with increased angiogenesis. However, the mechanism by which IL-17 mediates angiogenesis is undefined. Therefore, the pathologic role of CXCL1 and CXCL5 was investigated in arthritis mediated by local expression of IL-17, employing a neutralizing antibody to each chemokine. Next, endothelial chemotaxis was utilized to examine whether endothelial migration was differentially mediated by CXCL1 and CXCL5. Our results demonstrate that IL-17-mediated disease activity was not affected by anti-CXCL1 treatment alone. In contrast, mice receiving anti-CXCL5 demonstrated significantly reduced clinical signs of arthritis, compared to the mice treated with IgG control. Consistently, while inflammation, synovial lining thickness, bone erosion and vascularization were markedly reduced in both the anti-CXCL5 and combination anti-CXCL1 and 5 treatment groups, mice receiving anti-CXCL1 antibody had clinical scores similar to the control group. In contrast to joint FGF2 and VEGF levels, TNF-α was significantly reduced in mice receiving anti-CXCL5 or combination of anti-CXCL1 and 5 therapies compared to the control group. We found that, like IL-17, CXCL1-induced endothelial migration is mediated through activation of PI3K. In contrast, activation of NF-κB pathway was essential for endothelial chemotaxis induced by CXCL5. Although CXCL1 and CXCL5 can differentially mediate endothelial trafficking, blockade of CXCR2 can inhibit endothelial chemotaxis mediated by either of these chemokines. These results suggest that blockade of CXCL5 can modulate IL-17-induced inflammation in part by reducing joint blood vessel formation through a non-overlapping IL-17 mechanism.

**Keywords** IL-17-induced arthritis · CXCL1 · CXCL5 · Angiogenesis

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## Introduction

RA is an autoimmune disease in which angiogenesis can promote ingress of leukocytes, as well as pannus formation, thereby perpetuating inflammation and bone destruction [1]. Although RA was initially considered to be a TH-1-mediated disease, recent studies from experimental arthritis models indicate that TH-17 cells play a crucial role in the initiation and progression of the disease [2–5]. As such, the incidence and severity of collagen-induced arthritis (CIA) were significantly alleviated in IL-17-deficient mice, and post-onset blockade of IL-17 ameliorates CIA inflammation and joint destruction [6]. Further, local expression of

IL-17 exacerbates disease in the CIA [4] and K/BxN serum transfer arthritis models [3]. Not only can IL-17 amplify disease severity in experimental arthritis models, but its local expression can also mediate joint inflammation and synovial lining thickness in naive mice [3].

It has been shown that the proinflammatory activity of IL-17 is imparted by its ability to induce neutrophil ingression and granulopoiesis [7–9]. In studies using human neutrophils, migration induced by IL-17 was inhibited by a neutralizing antibody to IL-8, suggesting that IL-17-induced neutrophil migration is mediated through IL-8 production [10].

Previous studies have shown that IL-17-activated RA synovial tissue fibroblasts produce a number of CXC chemokines [11, 12] that are known to be neutrophil chemoattractant and proangiogenic. Further CXCL1 and CXCL5 mRNA transcripts are modulated by IL-17 through enhanced stabilization [12–14]. Chemokines such as CXCL1, 2, 3, 5 and 6, are corresponding ligands to CXCR2, and are important proangiogenic factors in RA joints [15–17] that can activate Matrigel tube formation and angiogenesis [18, 19]. Although blockade of CXCR1/CXCR2 in experimental arthritis models ameliorates joint inflammation by inhibiting adhesion and migration of neutrophils, the efficacy and the mechanism of the corresponding ligands are undefined [20–22].

Our recent studies demonstrate that IL-17 contributes to angiogenesis in RA since neutralization of IL-17 in RA synovial fluid or IL-17 receptor C (RC) on human microvascular endothelial cells (HMVECs) significantly reduces RA synovial fluid induced endothelial migration [23]. We also show that vascularity was increased in an IL-17-induced arthritis model [23].

In the current study, we examined the hypothesis that IL-17-mediated arthritis may be due to elevated chemokine levels that promote angiogenesis. To find these important factors, we screened IL-17-activated macrophages, RA synovial tissue fibroblasts and HMVECs for proangiogenic chemokine expression. Elevated proangiogenic chemokine expression was validated in the IL-17-induced arthritis model. Although expression of several factors was identified in these cell types/tissues, CXCL1 and CXCL5 were the most highly expressed in IL-17-activated RA synovial tissue explants and the experimental arthritis model. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17-mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17-mediated disease activity or neovascularization, while being capable of inhibiting CXCL1-mediated endothelial chemotaxis in vitro. The combination of anti-CXCL1 and anti-CXCL5 was not more effective than anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5-induced endothelial migration although down stream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF-$\kappa$B signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF-$\kappa$B mediated angiogenesis. In conclusion, these observations suggest that IL-17-mediated joint vascularization may be in part due to CXCL5 induction.

## Materials and methods

### Cell and tissue treatment for mRNA studies

The studies were approved by the Institutional Ethics Review Board and all donors gave informed written consent. Since the RA synovial tissues were recruited from the practices of orthopedic surgeons these samples are de-identified and therefore the disease severity and the treatment information is unavailable. RA synovial tissue fibroblasts were isolated from fresh RA synovial tissues, who fulfilled the American College of Rheumatology criteria for RA [24], by mincing and digesting in a solution of dispase, collagenase, and DNase [25–27]. Cells were used between passages 3–9. RA synovial tissue fibroblasts were treated with IL-17 (50 ng/ml) from 0 to 8 h for mRNA studies. Also, RA synovial tissue fibroblasts were either untreated or treated with IL-17 (50 ng/ml), TNF-$\alpha$ (10 ng/ml) or IL-17 plus TNF-$\alpha$ for 8 h. Monocytes were separated from buffy coats (Lifesource, Chicago, IL) obtained from healthy donors [26, 28]. Mononuclear cells, isolated by Histopaque (Sigma-Aldrich, St. Louis, MO) gradient centrifugation, were separated by countercurrent centrifugal elutriation. Monocytes were allowed to differentiate to macrophages as previously described [26, 28]. Macrophages were treated with IL-17 (50 ng/ml) from 0 to 8 h for mRNA studies. To determine IL-17-induced proangiogenic factors in RA synovial tissue, RA synovial tissue explants were activated with IL-17 (100 ng/ml) or PBS for 24 h. Thereafter, tissues were harvested and homogenized and protein levels of CXCL1, CXCL5, FGF2 and VEGF were determined by ELISA and results were shown as fold increase above RA synovial tissue explants treated with PBS. To define which signaling pathways mediate IL-17-induced CXCL1 or CXCL5 secretion, macrophages or RA fibroblasts were either untreated or incubated with DMSO or inhibitors to PI3K (LY294002; 10 $\mu$M), ERK (PD98059; 10 $\mu$M), JNK

(SP600125; 10 µM) or p38 (SB203580; 10 µM) for 1 h in serum free RPMI. Cells treated with DMSO or inhibitors were subsequently activated with IL-17 (50 ng/ml) for 24 h and the media was collected in order to quantify the levels of CXCL1 or CXCL5 employing ELISA.

## Real-time RT-PCR

Macrophages and RA synovial tissue fibroblasts were treated as mentioned in the figure legends and total cellular RNA was extracted using trizol (Invitrogen, Carlsbad, CA). Subsequently, reverse transcription and real-time RT-PCR was performed as previously described [23, 28, 29]. Relative gene expression was determined by the $\Delta\Delta C_t$ method normalized to GAPDH values, and results were shown as fold increase above 0 h and/or PBS treatment.

## Tissue homogenization

Mouse ankles were homogenized as described previously [29, 30] in 1 ml of Complete Mini protease-inhibitor cocktail homogenization buffer (Roche, Indianapolis, IN) on ice, followed by sonication for 30 s. Homogenates were centrifuged and filtered through a 0.45 µm pore size filter before quantifying the levels of various cytokines and chemokines by ELISA.

## Cytokine quantification

Mouse CXCL1, CXCL5, FGF2, VEGF, IL-1$\beta$, CCL2, CCL3, CCL5, CCL20, IL-1$\beta$, IL-6 and TNF-$\alpha$ (R&D Systems, Minneapolis, MN) ELISA kits were used according to the manufacturers' instructions.

## In vivo study protocol

The animal studies were approved by the Institutional Animal Care and Use Committee. Experiments were performed to determine the joint expression levels of CXCL1 and CXCL5 in an IL-17-induced arthritis model. For this purpose, 6-7-week-old C57BL/6 mice were injected intra-articularly with $10^7$ PFU adenoviral (Ad)-IL-17 or Ad-control [23, 27]. Ankles were harvested on days 4 and 10 post-Ad-IL-17 or Ad-control injection, and joint CXCL1, CXCL5, FGF2 (day 10) and VEGF (day 10) levels were quantified by ELISA. In a different set of studies, experiments were performed to determine whether CXCL1 and/or CXCL5 play a role in arthritis mediated by local IL-17 expression in mouse ankle joints. For this purpose, 6-7-week-old C57BL/6 mice were treated intraperitoneally with 30 µg (total of 210 µg was utilized in the course of treatment) of either IgG, monoclonal rat anti-mouse

CXCL1, monoclonal rat anti-mouse CXCL5 or both anti-CXCL1 and anti-CXCL5 antibodies (at the concentrations of 1–2.5 µg/ml, anti-CXCL1 and anti-CXCL5 are capable of neutralizing 50% (ND50) of mouse CXCL1 and CXCL5 (at 30 ng/ml); Leinco Technologies, St. Louis, Missouri) on days -4, -2, 0, 3, 5, 7 and 9 post-Ad injection with each group containing 10–12 mice. On day 0, Ad-IL-17 ($10^7$ PFU) was injected intra-articularly into the mouse ankle joints in each treatment group. Joint circumferences were measured on days 0, 3, 5, 7 and 10 post-Ad-IL-17 injection. On day 11, post-injection ankles were harvested for ELISA and immunohistochemical studies, and blood was collected by cardiac puncture to measure blood cell count using a HemaVet 850 complete blood counter (Drew Scientific, Waterbury, CT).

## Clinical assessments

Ankle circumferences were determined by measurement of two perpendicular diameters, the latero-lateral diameter and the antero-posterior diameter, using a caliper (Lange Caliper; Cambridge Scientific Industries). Circumference was determined using the following formula: circumference $= 2\Pi \times (\text{sqrt}(a^2 + b^2/2))$ [31, 32].

## Abs and immunohistochemistry

Mouse ankles were decalcified with ethylenediamine tetraacetic acid (Sigma, St. Louis, MO) in 10% formalin for 3 weeks, formalin fixed and paraffin embedded, and sectioned in the pathology core facility. Inflammation, synovial lining and bone erosion (based on a 0–5 score) [33] were determined using H&E-stained sections by a blinded observer (A.M.M.). Mouse ankles were immunoperoxidase-stained using Vector Elite ABC Kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen by the pathology core facility. Briefly, slides were deparaffinized in xylene for 15 min at room temperature, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in Proteinase K digestion buffer (Dako, Carpinteria, CA) for 5 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% $H_2O_2$ for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako, Carpinteria, CA). Nonspecific binding of antibodies to the tissues was blocked by pretreatment of tissues with Protein block (Dako). Tissues were incubated with Von willebrand factor (1:1,000 dilution; Dako) or control IgG antibody (Beckman Coulter, Brea, CA). Slides were counterstained with Mayer's hematoxylin and treated with lithium carbonate for bluing. Endothelial staining was scored on a 0–5 scale where 0 = no staining, 1 = few cells

stained, 2 = some (less than half) cells stained, 3 = around half of the cells were stained positively 4 = majority or more than half of the cells were positively stained, and 5 = all cells were positively stained. Data were pooled, the mean ± SEM was calculated and each slide was evaluated by a blinded observer (A.M.M.) [31, 32, 34, 35].

### Characterization of CXCL1 and CXCL5 activated signaling pathways in human microvascular endothelial cells (HMVECs)

HMVECs (passage 3–8) (Lonza, Walkersville, Maryland) were grown to 80% confluence in EGM-2 MV bullet kit (Lonza) and were incubated in endothelial basal medium (EBM) (Lonza) with 0% FBS for 2 h prior to treatment. Cells were then untreated or treated with CXCL1 or CXCL5 (20 ng/ml) for 5–65 min. Cell lysates were examined by Western blot analysis, as previously described [25, 26, 28]. Blots were probed with $I\kappa B$, phospho (p)-p38, pAKT and pERK (Cell Signaling; 1:1,000 dilution) overnight or probed with actin, p38, AKT or ERK (Sigma or Cell Signaling; 1:3,000 dilution) for 1 h.

### Examining the mechanism of CXCL1 and CXCL5-induced HMVEC migration

To examine chemotaxis, HMVECs were incubated in EBM (Lonza) with 0% FBS and no growth factors for 2 h before use. HMVECs ($2.7 \times 10^4$ cells/25 µl EBM with 0.1% FBS) from different treatments were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes (8 µm pore size; Nucleopore, Pleasant, CA) [23, 36]. To define which signaling pathway(s) mediated CXCL1 and CXCL5-induced HMVEC chemotaxis, HMVECs were incubated with DMSO or inhibitors to PI3K (LY294002; 1 and 5 µM), ERK (PD98059; 1 and 5 µM), p38 (SB203580; 1 and 5 µM) or NF-$\kappa$B (MG-132; 1 and 5 µM) at 37°C for 2 h, allowing endothelial cell attachment to the membrane [23]. The chamber was reinverted, and PBS, positive control VEGF (10 ng/ml; R&D Systems), CXCL1 (20 ng/ml; R&D Systems) or CXCL5 (20 ng/ml; R&D Systems) was added to the upper wells, and the chamber was further incubated for 2 h at 37°C. To examine whether CXCR2 is involved in CXCL1 and CXCL5-mediated HMVEC migration, HMVECs were incubated with antibody to CXCR2 (10 µg/ml, R&D systems; at 37°C for 2 h while cells were attaching to the membrane) and chemotaxis was examined in response to CXCL1 or CXCL5 (1 and 20 ng/ml; for 2 h at 37°C). Readings represent fold increase chemotaxis above cells migrating in response to PBS (cells were read

in three high power ×40 fields/well, averaged for each triplicate wells and subsequent values are shown as fold increase above PBS values from two different chemotaxis assays).

### Statistical analysis

The data was analyzed employing 1-way ANOVA followed by a post hoc two-tailed Student's $t$ tests for paired and unpaired samples. Values of $P < 0.05$ were considered significant.

## Results

### IL-17 induces the expression of CXCL1 and CXCL5 from cells present in the RA joint through activation of PI3K and/or ERK pathway and IL-17 synergizes with TNF-$\alpha$ in inducing the expression of CXCL1 and CXCL5 in RA synovial tissue fibroblasts

IL-17-induced downstream targets were determined employing RA synovial tissue fibroblasts, macrophages differentiated in vitro from monocytes and endothelial cells, because these cells are important in the pathogenesis of RA. We found that RA synovial tissue fibroblasts and peripheral blood differentiated macrophages that are activated with IL-17 express higher levels of CXCL1 and CXCL5 ($P < 0.05$) starting at 4 h or 6 h post-stimulation (Figs. 1a, 1d, 2a, 2d), compared to control treatment. Further, only the expression of CXCL1 was significantly upregulated in HMVECs activated by IL-17 as early as 2 h post-stimulation, compared to controls (data not shown). Our previous studies demonstrate that in macrophages and RA synovial tissue fibroblasts IL-17 signals through ERK, p38 and AKT while it only activates JNK pathway in RA synovial tissue fibroblasts [27]. To determine the mechanism by which IL-17 induces CXCL1 and CXCL5 production, these pathways were suppressed in RA synovial tissue fibroblasts and macrophages activated by IL-17. Our data demonstrate that inhibition of PI3K and ERK pathways suppress production of CXCL1 in macrophages and CXCL5 in both cell types (Figs. 1e, 2c, 2e). However, in RA fibroblasts only inhibition of PI3K was capable of reducing IL-17-mediated CXCL1 levels (Fig. 1c).

Interestingly, RA synovial tissue fibroblasts activated with IL-17 and TNF-$\alpha$ demonstrate significantly greater levels of CXCL1 (Fig. 1b) and CXCL5 (Fig. 2b), compared to cells activated with IL-17 or TNF-$\alpha$ alone. However, this synergistic effect was not detected in macrophages or when RA synovial tissue fibroblasts were stimulated with IL-17 and IL-1$\beta$ (data not shown). Our results suggest that CXCL1 and CXCL5 may be important
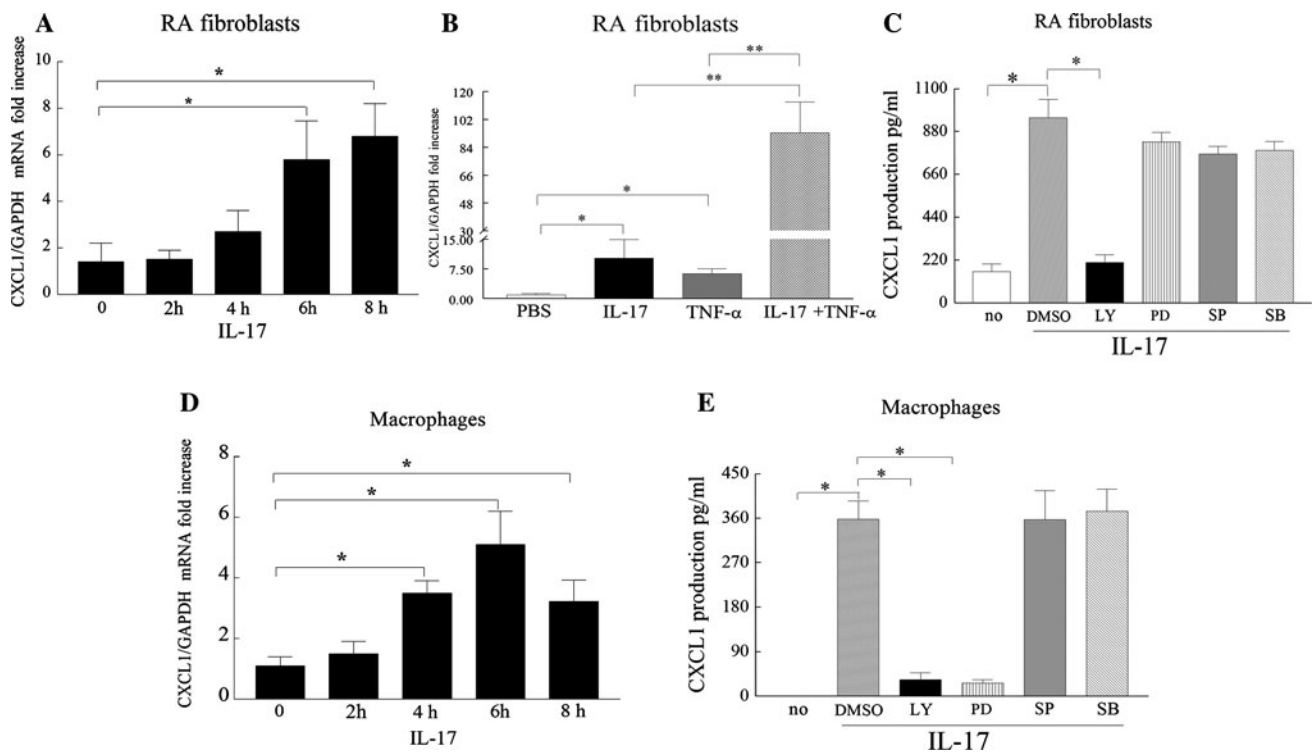
**Fig. 1** IL-17 induces production of CXCL1 in RA synovial fibroblasts and macrophages however, only in RA fibroblasts is CXCL1 expression synergistically induced by IL-17 and TNF-α. RA synovial tissue fibroblasts (**a**) and normal macrophages (**d**) were activated with IL-17 (50 ng/ml) for 0–8 h. Real-time RT-PCR was employed to identify CXCL1 (**a** and **d**) mRNA levels which were normalized to GAPDH. The results are presented as fold increase, compared with the 0 h time point (untreated cells). RA synovial tissue fibroblasts (**c**) and normal macrophages (**e**) were either untreated or incubated with DMSO or inhibitors to PI3K (LY294002; 10 μM), ERK (PD98059; 10 μM), JNK (SP600125; 10 μM) or p38 (SB203580; 10 μM) for 1 h. Thereafter cells treated with DMSO or inhibitors were subsequently activated with IL-17 (50 ng/ml) for 24 h and the media was collected from all conditions in order to quantify the levels of CXCL1 employing ELISA. **b** RA synovial tissue fibroblasts were either unstimulated or stimulated with IL-17 (50 ng/ml), TNF-α (10 ng/ml), or IL-17 plus TNF-α for 6 h. Cells were harvested, and CXCL1 mRNA levels were quantified by real-time RT-PCR which were normalized to GAPDH and presented as fold increase above PBS treatment (unstimulated cells). Values represent the mean ± SE. * Represents $P < 0.05$ and ** denotes $P < 0.01$, $n = 3$–5

downstream mediators expressed by RA synovial cells in response to IL-17 stimulation, and that TNF-α stimulation further promotes IL-17 induction of these chemokines.

### CXCL1 and CXCL5 are elevated in RA synovial tissue explants and IL-17-induced arthritis model

In order to determine the IL-17 modulated proangiogenic factors in RA synovial tissue explants and IL-17-induced arthritis model, levels of CXCL1, CXCL5, FGF2 and VEGF were quantified in IL-17 activated RA synovial tissue explants and/or IL-17-mediated arthritis ankles (harvested from day 10 post injection) and the data were demonstrated as fold increase above the control group (Fig. 3a, b). The results obtained from IL-17-induced arthritis model are similar to our finding in RA synovial tissue explants in that CXCL1 and CXCL5 are induced to a greater extent (40–10 fold increase in IL-17-induced arthritis ankles and 7–12 fold increase in RA explants

compared to the control group) compared to FGF2 (3–2 fold increase respectively), while VEGF was not significantly elevated in any of the mentioned models. Although in the IL-17-induced arthritis model the relative increase levels above Ad-control is greater for CXCL1 (40 fold) compared to CXCL5 (tenfold) the absolute joint concentrations for CXCL1 (1,600 pg/ml) and CXCL5 (1,520 pg/ml) are comparable in day 10 post injection (Fig. 3c, d). Based on these results we concluded that CXCL1 and CXCL5 may be important in IL-17 mediated pathogenesis in RA and this experimental arthritis model.

### Inhibition of CXCL5 but not CXCL1 ameliorates IL-17-induced arthritis

Experiments were performed to determine whether CXCL1 and/or CXCL5 play a role in arthritis mediated by local IL-17 expression in mice ankle joints. In mice locally injected with IL-17 (and IgG control), disease activity determined
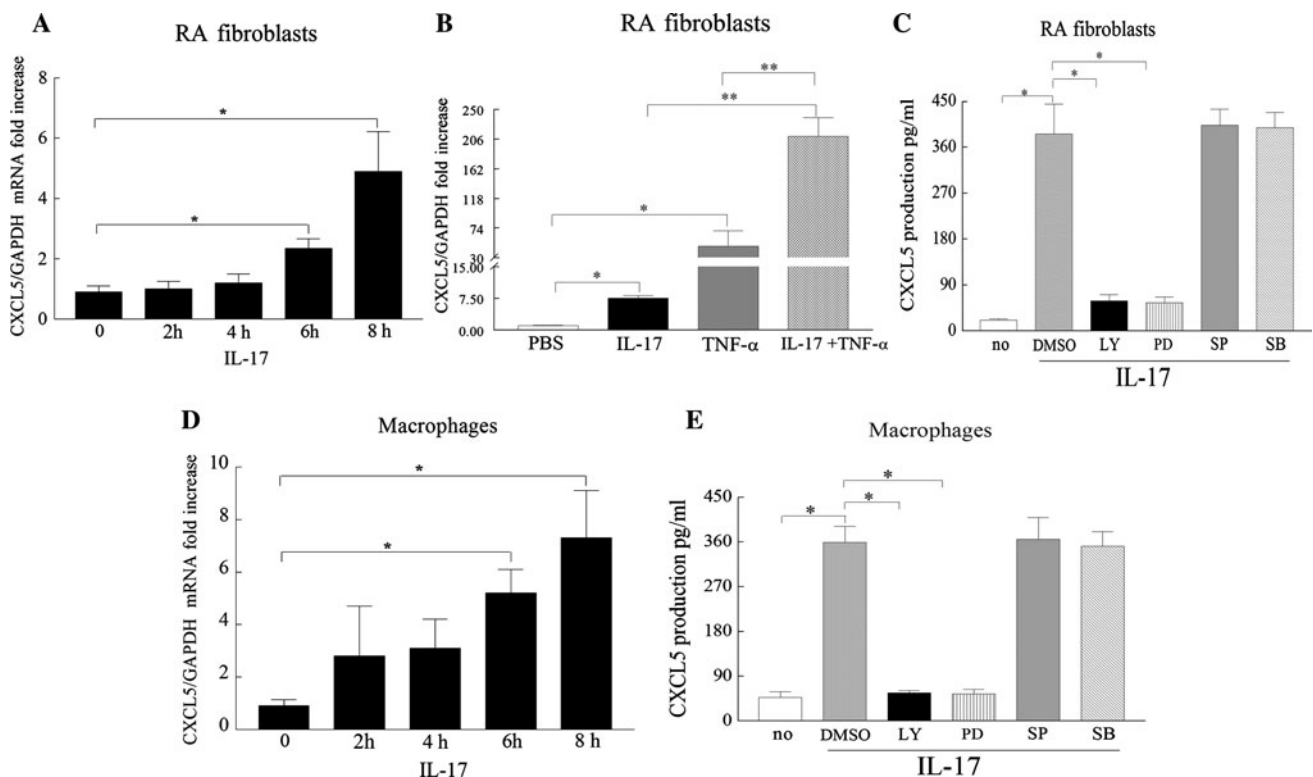
**Fig. 2** In RA synovial fibroblasts and macrophages, IL-17 induces production of CXCL5 however only in RA fibroblasts is CXCL5 expression synergistically induced by IL-17 and TNF-$\alpha$ stimulation. RA synovial tissue fibroblasts (**a**) and normal macrophages (**d**) were activated with IL-17 (50 ng/ml) for 0–8 h. Real-time RT-PCR was employed to identify CXCL5 (**a** and **d**) mRNA levels which were normalized to GAPDH. The results are presented as fold increase, compared with the 0 h time point (untreated cells). RA synovial tissue fibroblasts (**c**) and normal macrophages (**e**) were either untreated or incubated with DMSO or inhibitors to PI3K (LY294002; 10 μM), ERK (PD98059; 10 μM), JNK (SP600125; 10 μM) or p38 (SB203580; 10 μM) for 1 h. Thereafter cells treated with DMSO or inhibitors were subsequently activated with IL-17 (50 ng/ml) for 24 h and the supernatants were collected from all conditions in order to quantify the levels of CXCL5 by ELISA. **b** RA synovial tissue fibroblasts were either unstimulated or stimulated with IL-17 (50 ng/ml), TNF-$\alpha$ (10 ng/ml), or IL-17 plus TNF-$\alpha$ for 6 h. Cells were harvested and CXCL5 mRNA levels were quantified by real-time RT-PCR which were normalized to GAPDH and presented as fold increase above PBS treatment (unstimulated cells). Values represent the mean ± SE. * Represents $P < 0.05$ and ** denotes $P < 0.01$, $n = 3$–5

by ankle circumference began around day 3 and progressed through day 5, plateauing thereafter until the termination of the experiments on day 10 (Fig. 4a). The disease activity determined by ankle circumference was significantly lower in mice receiving anti-CXCL1 on days 3 and 5, compared to the control group. However, as the arthritis progressed there was no difference noted at later time points (days 7 and 10) (Fig. 4a). In vitro chemotaxis performed on endothelial cells demonstrated that the anti-CXCL1 antibody could markedly suppress CXCL1-induced endothelial migration while anti-CXCL5 antibody did not have any effect on this process (data not shown). Further, mice receiving anti-CXCL5 demonstrated significantly reduced clinical signs of arthritis at all time points, compared to the mice treated with IgG control ($P < 0.05$). The combination of anti-CXCL1 and anti-CXCL5 did not ameliorate IL-17-induced joint inflammation beyond the effect observed using anti-CXCL5 alone. Next, histological examination of

the joints was performed to determine the effect of treatment on inflammation, synovial lining and joint destruction. Consistent with the clinical data, histological analysis of the treatment groups demonstrated that inflammation, synovial lining thickening, and bone erosion were markedly reduced in the anti-CXCL5 and anti-CXCL1 and anti-CXCL5 treatment groups. In contrast, mice receiving anti-CXCL1 antibody had similar clinical scores compared to the control group (Fig. 4b, c). Our results demonstrate that CXCL5, and not CXCL1, plays an important role in IL-17-mediated arthritis.

**Anti-CXCL5 treatment downregulates proinflammatory mediators in IL-17-induced arthritis model**

To determine the role of CXCL1 and CXCL5 on IL-17-induced arthritis, proinflammatory mediators were quantified
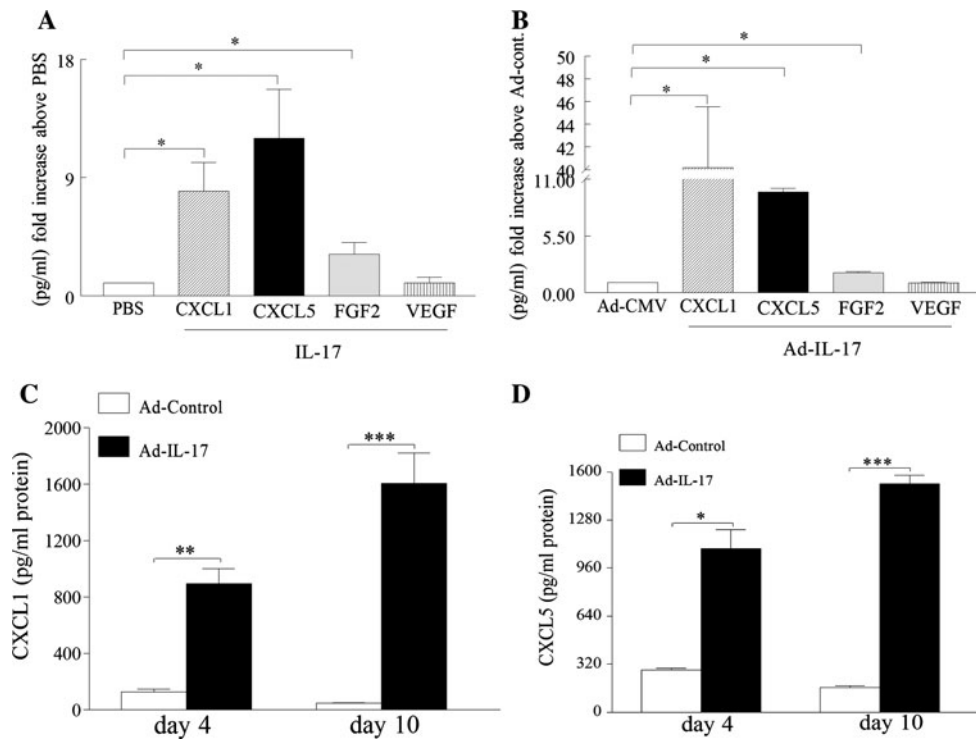
**Fig. 3** IL-17 increases the expression of CXCL1 and CXCL5 in RA synovial tissue explants and experimental arthritis model. **a** RA synovial tissue explants were treated with PBS or IL-17 (100 ng/ml), tissues were harvested after 24 h and levels of CXCL1, CXCL5, FGF2 and VEGF were quantified by ELISA and normalized to PBS values. **b** Ad-IL-17 or Ad-CMV control ($10^7$ PFU) was injected intra-articularly into C57/BL6 mice. Ankles were harvested on day 10 and in ankle joints. For this purpose, the effect of therapy was levels of CXCL1, CXCL5, FGF2 and VEGF were measured by ELISA and normalized to Ad-CMV. **c**, **d** Ankles from Ad-IL-17 and Ad-CMV treatment were harvested on days 4 and 10, and CXCL1 (**c**) and CXCL5 (**d**) levels were quantified by ELISA. Values are reported as mean ± SE. * Denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.005$, $n = 8$–10

in ankle joints. For this purpose, the effect of therapy was examined on joint TNF-$\alpha$, IL-6, IL-1$\beta$, CCL2, CCL3, CCL5, CCL20, CXCL2, FGF2 and VEGF protein levels. Our results demonstrate that mice receiving anti-CXCL5 or combination therapy had 40–50% lower levels of joint TNF-$\alpha$, compared to the control group (Fig. 4d). Joint CCL5 levels were also significantly (40–50%) reduced in IL-17-induced arthritis ankles receiving anti-CXCL5 or combination of anti-CXCL1 and 5, respectively (data not shown). Other joint proinflammatory mediators such as IL-6, IL-1$\beta$, CCL2, CCL3, CCL20, and CXCL2 were not affected by anti-CXCL5, anti-CXCL1 or combination treatments (data not shown). To demonstrate that the efficacy of anti-CXCL5 treatment is independent of the reduction of potent proangiogenic factors, joint FGF2 and VEGF were quantified in all four treatment groups. The data demonstrate that although levels of FGF2 but not VEGF are elevated in IL-17-induced arthritis model, ankles treated with anti-CXCL1 and anti-CXCL5 have similar levels of FGF2 (Fig. 5a) and VEGF (Fig. 5b) suggesting that anti-CXCL5 treatment can directly suppress IL-17-mediated angiogenesis. These results demonstrate that neutralization of CXCL5

modulates joint TNF-$\alpha$ and CCL5 levels in IL-17-mediated arthritis model.

### Anti-CXCL5 treatment reduces IL-17-induced vascularization

To determine the mechanism by which anti-CXCL5 ameliorates IL-17-induced arthritis, ankles were examined for joint vascularization. The data demonstrate that while levels of vascularization were similar in the IgG and the anti-CXCL1 treatment groups, anti-CXCL5 and the combination therapy had 40% fewer blood vessels (Fig. 5c, d). Our results may suggest that neutralization of CXCL5 can affect IL-17-induced arthritis through reduced blood vessel formation.

### Number of blood leukocytes, neutrophils and monocytes were unaffected in anti-CXCL1 and 5 treatments

To determine whether the IL-17-induced arthritis model could be affected by systemic treatment with anti-CXCL1
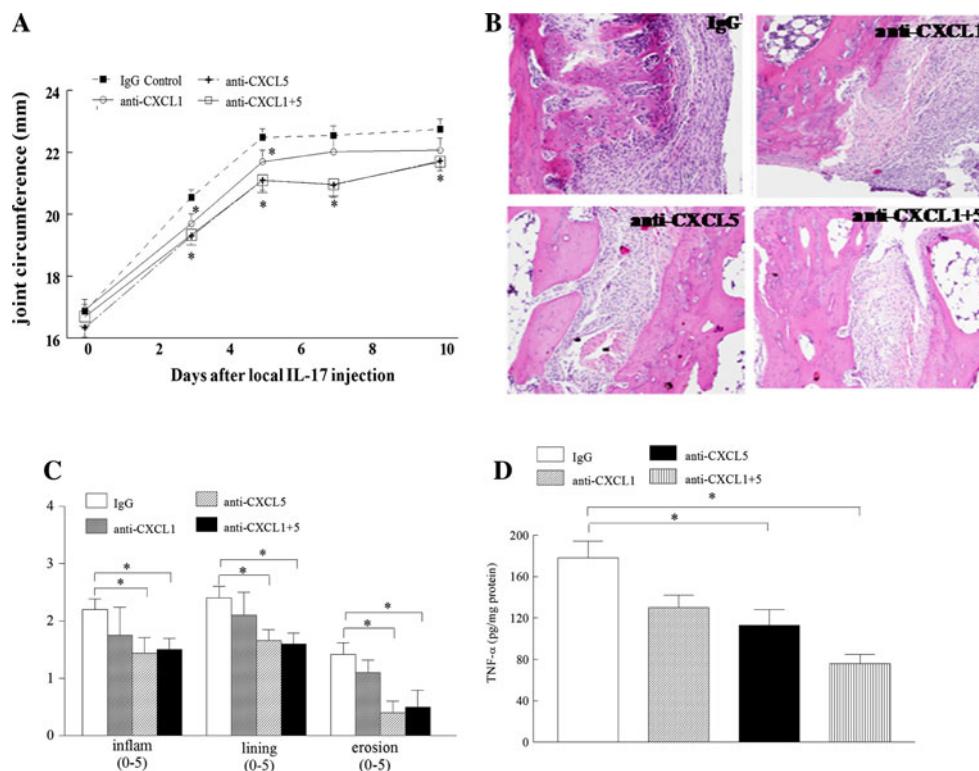
**Fig. 4** Neutralization of CXCL5 ameliorates IL-17-induced joint inflammation, synovial lining thickness, bone erosion and joint TNF-α levels. C57BL/6 mice were treated intraperitoneally with 30 μg (total of 210 μg was utilized over the course of treatment) IgG, anti-CXCL1, anti-CXCL5 or both anti-CXCL1 and 5 antibodies (Leinco Technologies) on days -4, -2, 0, 3, 5, 7 and 9 post-Ad injection. On day 0, Ad-IL-17 ($10^7$ PFU) was injected intra-articularly into the mouse ankle joint, and the joint circumference (**a**) was measured on days 0, 3, 5, 7 and 10 post-Ad-IL-17 injection and each experimental group consisted of 10–12 mice. **b, c** Inflammation, synovial lining and bone erosion (based on a 0–5 score) were determined using H&E-stained sections by a blinded observer, $n = 10$ ankles. Changes in the levels of joint TNF-α (**d**) were measured in ankle homogenates obtained from different treatment groups by ELISA and were normalized by protein concentration, $n = 7$–9 ankles. Values demonstrate mean ± SE. * Denotes $P < 0.05$

and/or anti-CXCL5, the number of leukocytes, neutrophils and monocytes were measured in mouse whole blood. Interestingly, all treatment groups had similar numbers of white blood cells, neutrophils and monocytes (Fig. 6a), in contrast to lower levels of joint neutrophils in the anti-CXCL5 and combination therapy (data not shown). These results suggest that although the number of circulating cells was unchanged in the anti-CXCL5 and combination therapy groups, fewer cells migrated into these IL-17-mediated arthritis joints (as shown in H&E staining in Fig. 4b, c).

CXCL1 and CXCL5 mediate endothelial migration through CXCR2 ligation

To demonstrate whether CXCR2 ligation is involved in CXCL1 and CXCL5 function, CXCR2 on endothelial cells was blocked employing anti-CXCR2 neutralizing antibody and subsequently endothelial chemotaxis was examined in response to CXCL1 and CXCL5. Results from these experiments demonstrate that neutralization of CXCR2 on HMVECs significantly decreases CXCL1 (50%) and

CXCL5 (40–50%)—mediated endothelial migration (Fig. 6b) suggesting that both chemokines require CXCR2 ligation in order to mediate chemotaxis despite them signaling through different signaling pathways.

CXCL1 and CXCL5 induce endothelial migration through different signaling pathways

To address the different efficacy of blocking CXCL1 and CXCL5 in IL-17 experimental arthritis model we examined the mechanism by which these chemokines induce endothelial migration. We found that in HMVECs, CXCL1 signals through PI3K (35 min) and ERK (35 min) however this chemokine was unable to activate NF-κB or p38 signaling pathways (Figs. 7a). CXCL5 stimulation of HMVECs results in activation of NF-κB pathway (65 min) only (Figs. 7c). To demonstrate the mechanism by which CXCL1 and CXCL5 mediate HMVEC migration, inhibitors to these pathways were employed in in vitro chemotaxis. Interestingly while inhibition of PI3K suppresses CXCL1-induced HMVEC migration, chemotaxis mediated
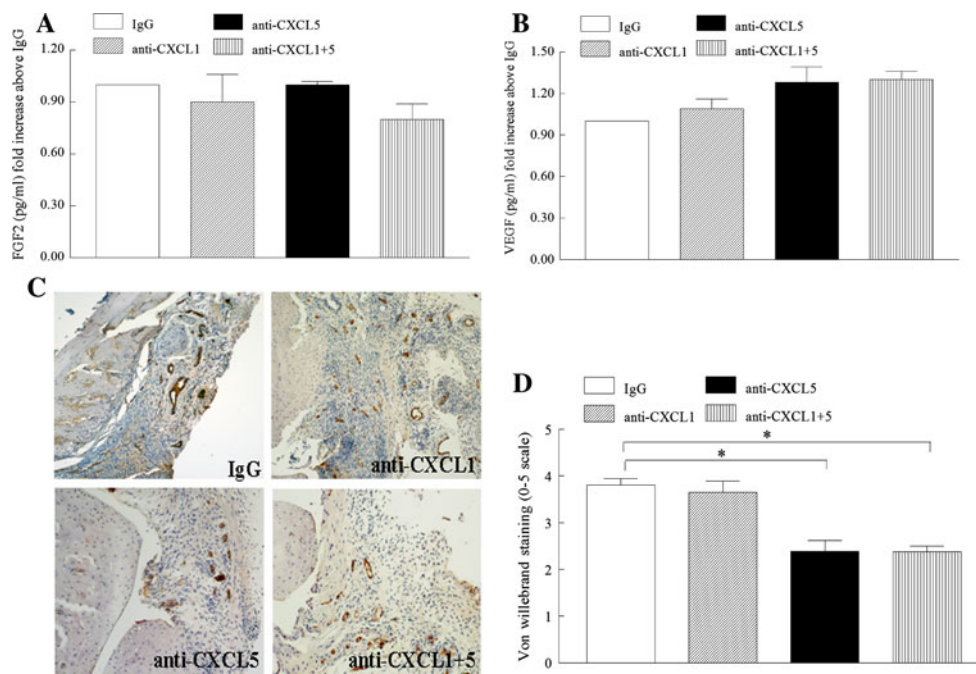
Fig. 5 Levels of joint FGF2 and VEGF were unaffected by anti-CXCL5 and combination therapy and anti-CXCL5 therapy downregulates IL-17-induced joint vascularization. Changes in the levels of joint FGF2 (a) and VEGF (b) were measured in ankle homogenates obtained from different treatment groups by ELISA and the data is presented as fold increase above the control IgG treatment, $n = 7–9$ ankles. IL-17-induced arthritis ankles treated with IgG, anti-CXCL1, anti-CXCL5 or the combination therapy were harvested on day 11 and were immunostained with Von willebrand factor (endothelial marker) (c) (original magnification × 200). Quantification of endothelial (d) staining from IL-17-induced arthritis ankles harvested on day 11, $n = 8–9$ ankles. Values demonstrate mean ± SE. * Denotes $P < 0.05$

by CXCL5 was reduced through NF-$\kappa$B inhibition (Figs. 7b, d). These results suggest that endothelial migration is differentially regulated by CXCL1 and CXCL5.

## Discussion

In this study, we show that CXCL1 and CXCL5 are important downstream mediators of IL-17 in RA synovial cells, RA synovial tissue explants and the IL-17-induced arthritis model. Neutralization of CXCL5, but not CXCL1, ameliorates joint inflammation, bone destruction and vascularization mediated by local expression of IL-17. The differential effect of CXCL1 and CXCL5 blockade in IL-17-induced arthritis model may be due to CXCL5 mediating endothelial migration through a nonoverlapping pathway with IL-17 and CXCL1 despite both chemokines ligation to CXCR2. These results suggest that differential regulation of angiogenesis by CXCL5 can suppress IL-17-induced joint inflammation.

To determine IL-17 downstream targets, RA synovial tissue fibroblasts, macrophages and HMVECs were employed. We found that genes highly induced by IL-17 were potent proangiogenic factors. We also demonstrated

that while IL-17-stimulated RA synovial tissue fibroblasts and macrophages express elevated levels of CXCL1 and CXCL5, activated HMVECs demonstrated only higher CXCL1 expression (data not shown). Consistent with our data, others have shown that CXCL1 and CXCL5 expression levels are significantly elevated in IL-17-activated preosteoblast cell line MC3T3-E1 [12]. In macrophages, CXCL1 and CXCL5 are similarly induced by IL-17 through PI3K and ERK pathways. However in RA fibroblasts, CXCL1 and CXCL5 production is differentially regulated by IL-17. Consistently, others have shown that activation of PI3K pathway plays an important role in IL-17-induced CXC chemokine expression in bronchial epithelium cells [37]. In contrast, previous studies demonstrate that IL-17-mediated CXCL1 and 2 expression in RA fibroblasts is suppressed by inhibition of p38 pathway [38]. The inconsistency in the data may be due to differences in passage number, growth condition, methods employed for quantifying mRNA levels (authors determined mRNA after 24 h treatment) [38] or patient treatment employed in the donated RA synovial tissues.

Interestingly, in RA synovial tissue fibroblasts, IL-17 and TNF-$\alpha$, but not IL-1$\beta$, synergize in inducing the expression of CXCL1 and CXCL5. The amplifying effect of IL-17 and TNF-$\alpha$ in RA synovial tissue fibroblasts was

**A**

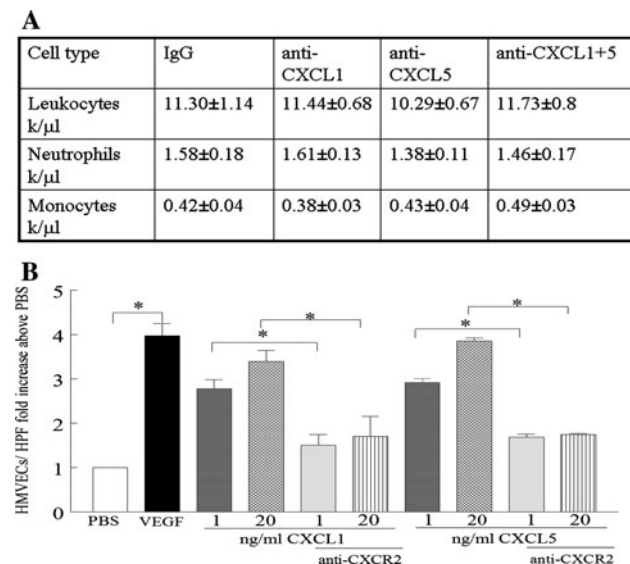| Cell type | IgG | anti-CXCL1 | anti-CXCL5 | anti-CXCL1+5 |
|---|---|---|---|---|
| Leukocytes k/μl | 11.30±1.14 | 11.44±0.68 | 10.29±0.67 | 11.73±0.8 |
| Neutrophils k/μl | 1.58±0.18 | 1.61±0.13 | 1.38±0.11 | 1.46±0.17 |
| Monocytes k/μl | 0.42±0.04 | 0.38±0.03 | 0.43±0.04 | 0.49±0.03 |



Fig. 6 Anti-CXCL5 treatment did not affect the circulating number of leukocytes, neutrophils and monocytes in IL-17-mediated arthritis model and CXCL1 and CXCL5 mediate endothelial migration through CXCR2 ligation. **a** On day 11 blood was collected by cardiac puncture of IL-17-induced arthritis ankles treated with IgG, anti-CXCL1, anti-CXCL5 or the combination therapy to measure blood cell count using a HemaVet 850 complete blood counter. Values are shown in thousands of cells per microliter of blood (k/μl, $n = 10$–12 mice). **b** HMVECs incubated with antibody to CXCR2 (10 μg/ml, R&D systems) were kept at 37°C for 2 h while cells were attaching to the membrane and chemotaxis was examined in response to CXCL1 and CXCL5 (1 and 20 ng/ml; for 2 h at 37°C), $n = 2$. Values represent fold increase chemotaxis above cells migrating in response to PBS shown as mean ± SE of two experiments in triplicate. * Represents $P < 0.05$

very specific to proangiogenic chemokines, and the same effect was not detected for monocyte chemokines such as CCL2 (data not shown). Additionally, this synergistic effect on CXCL1 and CXCL5 was not noted when macrophages or HMVECs were activated with IL-17 and TNF-α, suggesting that the effect was specific to RA synovial tissue fibroblasts. Consistently, others have shown that in RA fibroblasts, IL-17 can synergize with TNF-α and IL-1β in inducing the production of CCL21 [39]. In nonmyeloid cells IL-17-induced stabilization of CXCL1 is independent of AUUUA motif [40] however activator of NF-kappaB1 protein (Act1) is required for this process[41]. While TNF-α mediated transcription of CXC chemokines is driven by NF-κB, this process is modulated by IL-17 through stabilizing the mRNA in an Act1 dependent manner [41]. Hence the synergy between TNF-α and IL-17 may reflect their independent effects on CXC chemokines.
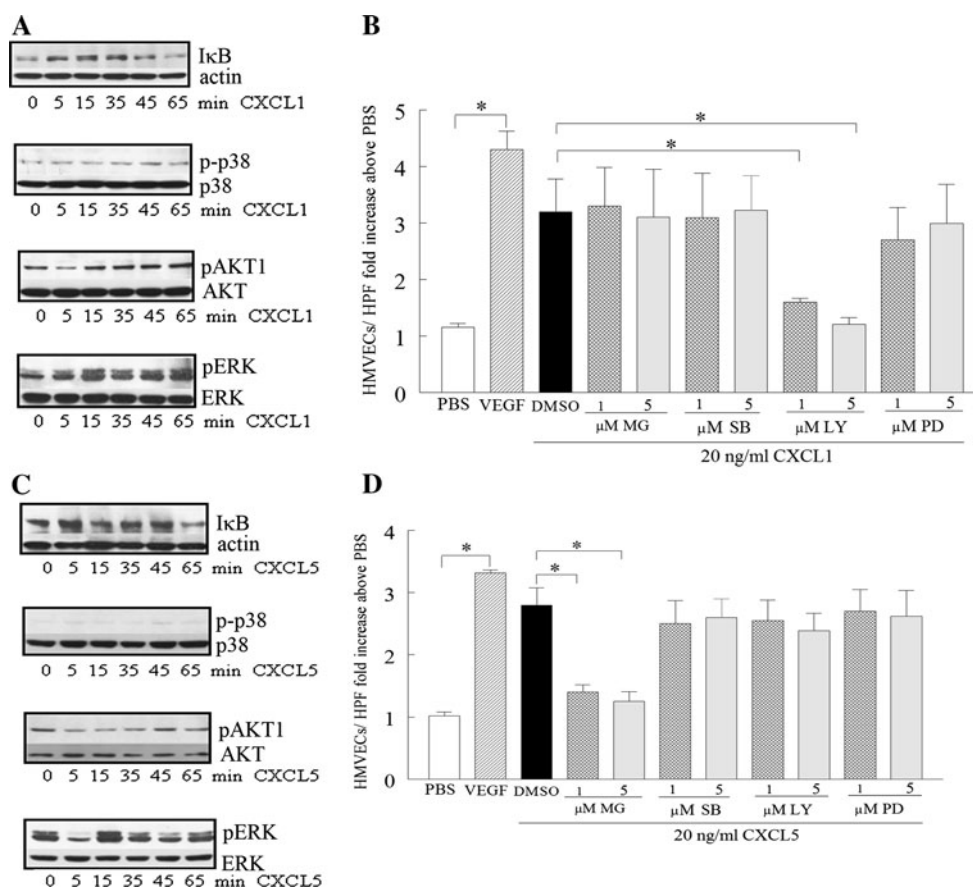
Since both CXCL1 and CXCL5 were significantly elevated in RA synovial tissue explants and IL-17-induced arthritis model to a greater extent than other proangiogenic factors such as FGF2 and VEGF, we asked whether neutralization of one or both of these chemokines could

alleviate joint inflammation mediated by local expression IL-17. We found that neutralization of CXCL1 reduced joint inflammation initially on days 3 and 5 post-IL-17 local expression, but was unable to reduce the joint swelling at later time points when arthritis was established. However, CXCL1-mediated endothelial chemotaxis in vitro was markedly reduced by anti-CXCL1 antibody (data not shown). In contrast to anti-CXCL1 treatment, anti-CXCL5 therapy effectively reduced joint inflammation, lining thickness and bone erosion throughout the disease course in the IL-17-induced arthritis model. The combination of anti-CXCL1 and CXCL5 did not ameliorate IL-17-induced joint inflammation beyond the effect observed using anti-CXCL5 alone, indicating that the clinical efficacy was due to blockade of joint CXCL5. Despite elevated levels of FGF2 in IL-17-induced arthritis model, levels of this proangiogenic factor were unaffected by anti-CXCL5 treatment indicating that the efficacy of anti-CXCL5 treatment is directly mediated through CXCR2 ligation. As demonstrated by endothelial chemotaxis data, both CXCL1 and CXCL5 bind to CXCR2, however our results suggest that ligation of these ligands may differentially activate down stream signaling pathways.

Since blockade of CXCL5, but not CXCL1, reduced IL-17 joint vascularization we next examined the mechanism by which these chemokines induce endothelial migration. Interestingly in HMVECs, CXCL1 stimulation resulted in PI3K and ERK signaling whereas only NF-κB pathway was activated by CXCL5 in these cells. Other studies have shown that while stimulation with CXCL1 can phosphorylate ERK1/2 pathway [42–44], activation with CXCL5 is involved with PI3K and NF-κB signaling pathways [45]. We further demonstrate that similar to IL-17 [23], CXCL1 mediated HMVEC migration is through PI3K activation. In contrast, inhibition of NF-κB suppresses endothelial chemotaxis induced by CXCL5. Perhaps inhibition of CXCL1 is ineffective in reducing joint inflammation since IL-17 is present in the mouse ankles (1,200 pg/mg and 400 pg/mg on days 4 and 10 post injection respectively [27]) and can induce angiogenesis through the same mechanism. In line with our finding others have shown that CXCL1 and 5 can differentially modulate monocyte arrest and migration [46], suggesting that ligands binding to the same receptor can have distinct functions through activating different signaling intermediates.

Reduction in joint TNF-α levels in the anti-CXCL5 and combination therapy may be due to the fact that IL-17-induced joint pathology is abrogated in TNF-α deficient mice, indicating that in this model TNF-α is required [3]. It has also been shown that IL-17 can directly modulate TNF-α secretion from macrophages [47]. Hence, suppressing IL-17-induced inflammation may reduce TNF-α production from macrophages in the synovial lining and sublining.

**Fig. 7** CXCL1 and CXCL5 induce endothelial migration through activating different signaling pathways. In order to determine the mechanism by which (**a**) CXCL1 and (**c**) CXCL5 activate HMVECs, cells were stimulated with these chemokines (20 ng/ml) for 0–65 min, and the cell lysates were probed for IκB, p-p38, p-AKT, and pERK and/or equal loading controls. To determine signaling pathways associated with **b** CXCL1 and **d** CXCL5-induced HMVEC migration, cells were treated with DMSO or inhibitors to NF-κB (MG-132; 1 and 5 μM), p38 (SB203580; 1 and 5 μM), PI3K (LY294002; 1 and 5 μM) and ERK (PD98059; 1 and 5 μM) 2 h in the Boyden chamber, $n = 2$. Values represent fold increase chemotaxis above cells migrating in response to PBS shown as mean ± SE of two experiments in triplicate. * Represents $P < 0.05$

Further, both TNF-α and IL-17 synergize in inducing the expression of CXCL5 from RA fibroblasts. Therefore, neutralization of CXCL5 may have a negative feed back regulation on joint TNF-α concentrations. When RA synovial tissue fibroblasts, macrophages and HMVECs were screened for IL-17 downstream targets, CCL5 was undetected (data not shown). Therefore, reduction in joint CCL5 concentration in anti-CXCL5 and combination therapy treatment groups may be due to reduced TNF-α levels, since CCL5 expression is known to be modulated by TNF-α in RA synovial tissue fibroblasts [48, 49].

In conclusion, anti-CXCL5 treatment ameliorates IL-17-mediated arthritis by down regulating TNF-α and joint vascularization through an IL-17 nonoverlapping mechanism. These data support angiogenesis as an important mechanism by which IL-17 contributes to RA pathogenesis, further supporting IL-17 as a potential therapeutic target in RA.

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Standards** All experiments performed comply with the current laws of United States of America.

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# TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF-α Levels

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**The innate immune system plays an important role in rheumatoid arthritis (RA) pathogenesis. Previous studies support the role of TLR2 and 4 in RA and experimental arthritis models; however, the regulation and pathogenic effect of TLR5 is undefined in RA. In this study, we show that TLR5 is elevated in RA and osteoarthritis ST lining and sublining macrophages and endothelial cells compared with normal individuals. Furthermore, expression of TLR5 is elevated in RA synovial fluid macrophages and RA peripheral blood monocytes compared with RA and normal peripheral blood in vitro-differentiated macrophages. We also found that TLR5 on RA monocytes is an important modulator of TNF-α in RA synovial fluid and that TLR5 expression on these cells strongly correlates with RA disease activity and TNF-α levels. Interestingly, TNF-α has a feedback regulation with TLR5 expression in RA monocytes, whereas expression of this receptor is regulated by IL-17 and IL-8 in RA macrophages and fibroblasts. We show that RA monocytes and macrophages are more responsive to TLR5 ligation compared with fibroblasts despite the proinflammatory response being mediated through the same signaling pathways in macrophages and fibroblasts. In conclusion, we document the potential role of TLR5 ligation in modulating transcription of TNF-α from RA synovial fluid and the strong correlation of TLR5 and TNF-α with each other and with disease activity score in RA monocytes. Our results suggest that expression of TLR5 may be a predictor for RA disease progression and that targeting TLR5 may suppress RA.** *The Journal of Immunology*, 2012, 189: 475–483.

R heumatoid arthritis (RA) is a chronic autoimmune disorder in which the innate immune system plays an important role (1, 2). TLRs are pattern recognition receptors that are present in a number of cells and tissues, which recognize pathogen-associated molecular patterns (PAMPs) or endogenous ligands (3).

Previous studies demonstrate that expression of TLR2 and TLR4 is elevated in RA peripheral blood (PB) monocytes as well as in RA synovial fluid and synovial tissue (ST) macrophages (4–7). Increased TLR2 and TLR4 expression was detected in RA compared with osteoarthritis (OA) ST fibroblasts (8). Further, the data obtained from experimental arthritis models strongly support the role of TLR2 and TLR4 in streptococcal cell wall arthritis (9, 10) while TLR4 has been implicated in collagen-induced arthritis (11)

as well as in the IL-1RA$^{-/-}$ model (11, 12). However, the role of TLR5 in RA and murine models of RA is undefined.

TLR5 is expressed on a variety of cell types such as epithelial cells, neutrophils, monocytes, macrophages, and mast cells and is the receptor for the bacterial structural protein flagellin (13). Flagellin signaling via TLR5 is dependent on MyD88 and IL-1R–associated kinase 1 (14, 15) and subsequent activation of NF-κB, MAPK, and PI3K pathways (16–18). As with other TLR agonists, flagellin has been shown to induce dendritic cell maturation and activation (19), thereby promoting lymphocyte migration to secondary lymphoid sites (20). Others have shown that spontaneous neutrophil apoptosis is delayed by flagellin through induction of Mcl-1 and inhibition of caspase-3 (21). What remains unclear is whether TLR5 is present in RA synovium and whether ligation of this receptor plays a role in RA pathogenesis.

In this study, to our knowledge, we demonstrate for the first time that TLR5 is elevated in RA and OA ST lining and sublining macrophages and endothelial cells compared with normal (NL) controls. Consistently, our data demonstrate that TLR5 expression is greatly elevated in RA synovial fluid macrophages and PB monocytes compared with their NL counterparts. In RA monocytes, patients with higher expression of TNF-α expressed elevated levels of TLR5 and the concentration of both of these factors strongly correlated with increased disease activity score (DAS28). The role of TLR5 expression in RA pathogenesis was documented when the blockade of TLR5 on monocytes significantly reduced synovial fluid-mediated TNF-α transcription by 80%. Interestingly, we demonstrate a feedback modulation between TNF-α production and TLR5 ligation and expression in RA monocytes. Although in RA macrophages, TLR5 expression is induced by IL-17 and IL-8, it is significantly reduced by TLR4 ligation in both RA monocytes and macrophages. Higher expression of TLR5 was detected in RA compared with NL fibroblasts, which was upreg-

ulated by a variety of inflammatory factors excluding LPS. Hence, our data demonstrate the expression of TLR5 in RA and further documents its importance in RA disease activity and TNF-α modulation.

## Materials and Methods

### Abs and immunohistochemistry

The studies were approved by the Institutional Review Board, and all donors gave informed written consent. Because the RA ST are recruited from the practices of orthopedic surgeons these samples are de-identified; therefore the disease severity and the treatment information is unavailable. RA, OA, and NL ST were formalin fixed, paraffin embedded, and sectioned in the pathology core facility. ST were immunoperoxidase-stained using Vector Elite ABC kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Briefly, slides were deparaffinized in xylene for 15 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by incubating slides in proteinase K digestion buffer (DakoCytomation, Carpinteria, CA) for 10 min at room temperature. Tissues were incubated with Abs to human TLR5 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) or an IgG control Ab (Beckman Coulter, Brea, CA). Slides were evaluated by blinded observers (22–25) (A.M.M. and M.V.V.). Tissue sections were scored for lining, sublining macrophages and endothelial cell staining on a 0–5 scale (26, 27). Scored data were pooled, and the mean ± SEM was calculated in each data group. To demonstrate location of TLR5 in RA ST serial tissue sections were stained with anti-TLR5 (1:50; Santa Cruz Biotechnology), anti-CD68 (1:100; Vector Laboratories), and anti-von Willebrand factor (VWF) (1:1000; Vector Laboratories) Abs. To localize TLR5 to macrophages in RA ST, slides were deparaffinized and unmasked as mentioned above. Using an Invision G2 kit (DakoCytomation), RA ST were stained with anti-TLR5 Ab (1:50 dilution; Santa Cruz Biotechnology) using diaminobenzidine (brown staining) as a chromogen. Thereafter, tissues were blocked (double staining blocker included in the Invision G2 kit) and stained with anti-CD68 Ab (1:100 dilution; DakoCytomation) using Fast red (red staining) as a chromogen following the manufacturers' instructions (DakoCytomation).

### RA patient population

RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology (28). PB was obtained from 44 women and 4 men (mean age, 53.7 ± 2.7 y). At the time of treatment, patients were receiving no treatment ($n = 7$), taking nonbiological disease-modifying antirheumatic drugs (DMARDs) (methotrexate, leflunomide, and sulfasalizine azathioprine) alone ($n = 5$), taking DMARDs plus hydroxychloroquine ($n = 9$), taking DMARDs plus prednisone ($n = 5$), taking DMARDs plus rituximab ($n = 3$), taking DMARDs plus hydroxychloroquine plus minocycline ($n = 2$), or taking a TNF-α inhibitor alone ($n = 6$), with a DMARD ($n = 8$), a DMARD plus prednisone ($n = 1$), or with a DMARD plus hydroxychloroquine and prednisone ($n = 2$). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. Maximum number of patients was 48; however, please refer to the figure legends for exact number of patients in each experiment.

### Cell isolation, culture, and procedures

NL and RA PB and RA synovial fluid mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO) as described previously (29, 30). Monocytes/macrophages were isolated from NL and RA PB or RA synovial fluid using a negative selection kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's instructions (26, 27). Monocytes were subsequently differentiated to macrophages by culturing in RPMI 1640 medium containing 20% FBS for 7 d.

### Quantification of chemokines and cytokines

Human TNF-α, IL-6, and CCL2 (R&D Systems, Minneapolis, MN) ELISA kits were used according to the manufacturer's instructions.

### Isolation of RA ST fibroblasts

ST fibroblasts were isolated from fresh RA ST by mincing and digestion in a solution of dispase, collagenase, and DNase (30). Cells were used

between passages 3 and 9 and cultured in DMEM containing 10% FBS, and cell purity was validated by CD90 staining.

### Cell treatment

RA PB monocytes and in vitro-differentiated macrophages or RA ST fibroblasts were treated with polyinosinic-polycytidylic acid (poly I:C) (10 ng/ml, only in RA monocytes; InvivoGen, San Diego, CA), LPS (10 ng/ml; Sigma-Aldrich), IL-1β (10 ng/ml; R&D Systems), TNF-α (10 ng/ml; R&D Systems), IL-17 (50 ng/ml; R&D Systems), IL-6 (10 ng/ml; R&D Systems), IL-8 (10 ng/ml; R&D Systems), or RA synovial fluid (10%). Cells were harvested after 6 h, and the TLR5 mRNA levels were quantified by real-time RT-PCR. RA ST fibroblasts, RA PB monocytes, and differentiated macrophages were treated with flagellin Ultra pure (10 and 100 ng/ml) (endotoxin levels < 50 EU/mg) (InvivoGen), cells (6 h; for real-time RT-PCR) or conditioned media (24 h; for ELISA) were harvested following treatment, and TNF-α, IL-6, and CCL2 mRNA production was quantified. In a different experiment, RA monocytes from six different patients were treated with anti-TLR5 Ab or IgG (10 μg/ml; InvivoGen) for 1 h prior to being treated with RA synovial fluid (10%; $n = 6$) for 6 h. To demonstrate that reduction of RA synovial fluid-mediated TNF-α levels are due to blockade of TLR5 and not to the necrotic effect of this Ab in RA monocytes, cells were pretreated with anti-TLR5 Ab or IgG control 1 h prior to treating the cells with PBS or flagellin for 6 h. Subsequently, the TNF-α mRNA levels were quantified by real-time RT-PCR for experiments performed for Fig. 3A and 3B.

### Real-time RT-PCR

Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from the different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine TLR5, TNF-α, IL-6, and CCL2 expression levels as described previously (29–31). Relative gene expression was determined by the $\Delta\Delta C_t$ method, and results were expressed as fold increase above conditions indicated in the figure legends.

### Flow cytometry

To determine TLR5$^+$ cells, NL and RA monocytes and differentiated macrophages were washed with FACS buffer (5% FBS in PBS). Thereafter, cells were blocked with 50% human serum and 0.5% BSA. Cells were then stained with PE-conjugated anti-TLR5 (Imgenex, San Diego, CA) and FITC-labeled anti-CD14 (BD Immunocytometry, Franklin Lakes, NJ) or isotype control Abs (BD Pharmingen). Percent TLR5$^+$ cells were identified as those that were CD14$^+$TLR5$^+$. Because of limited access to RA synovial fluid macrophages, these cells were not included in the FACS analysis.

### Flagellin signaling pathways in RA macrophages or RA fibroblasts

RA ST fibroblasts and macrophages ($2 \times 10^6$/ml) were untreated or treated with flagellin (100 ng/ml) for 0–65 min. Cell lysates were examined by Western blot analysis (30). Blots were probed with p-ERK, p-p38 MAPK, p-AKT1, p-JNK (1:1000 dilution; Cell Signaling Technology), or degradation of IκB (1:3000 dilution; Sigma-Aldrich) overnight or probed with ERK, p38, AKT, and JNK or actin (1:3000 dilution; Cell Signaling Technology or Sigma-Aldrich).

### Inhibition of the signaling pathways in RA ST fibroblasts and macrophages

To define which signaling pathways mediate flagellin-induced CCL2 secretion, RA macrophages and fibroblasts were incubated with DMSO or 10 μM inhibitors to p38 (SB203580), ERK (PD98059), JNK (SP600125), PI3K (LY294002), or NF-κB (MG-132) for 1 h in RA-differentiated macrophages or fibroblasts. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the media were collected to quantify the levels of CCL2 using ELISA.

### Statistical analysis

The data were analyzed using one-way ANOVA, followed by a post hoc two-tailed Student $t$ test for paired and unpaired samples. In RA monocytes, TLR5 and TNF-α mRNA expression was correlated with each other using the $\Delta\Delta C_t$ method. Further TLR5 or TNF-α mRNA mass was normalized to its GAPDH mass, and values were correlated with DAS28 score using linear regression analysis in RA monocytes. The $p$ values < 0.05 were considered significant.

## Results

### TLR5 elevated in RA and OA ST

To characterize the expression pattern of TLR5 in RA and OA compared with controls, ST were stained with Abs against TLR5. We found that both in RA and OA, TLR5 immunostaining was significantly higher on ST lining and sublining macrophages and endothelial cells compared with NL ST (Fig. 1A–D). Consistently, TLR5 staining was colocalized to RA ST CD68^{+} (Fig. 1E, 1F, 1H) and VWF^{+} cells (Fig. 1E, 1G). Although previous studies demonstrate that fibroblasts in the lining and macrophages in the lining and sublining express TLR2 and 4 (32), expression of these receptors has not been reported in endothelial cells. Therefore, TLR5 may be a member of the TLR family that is uniquely elevated on RA and OA endothelial cells.

### RA synovial fluid macrophages and RA PB monocytes express upregulated levels of TLR5

Because TLR5 immunostaining was elevated in RA ST lining and sublining macrophages, we asked whether mRNA and/or cell surface expression of this receptor was increased in RA synovial fluid macrophages compared with RA and NL PB monocytes or differentiated macrophages. Using microarray analysis, TLR5 was identified as one of the genes (3.2-fold higher; $p = 1.58 \times 10^{-10}$) that was greatly increased in RA synovial fluid macrophages compared with NL macrophages. These results were confirmed when real-time RT-PCR demonstrated that the expression of TLR5 was elevated 9- and 35-fold in RA synovial fluid macrophages compared with RA and normal PB-differentiated macrophages, respectively (Fig. 2A). Furthermore, levels of TLR5 were 7- and 3-fold greater in RA PB monocytes compared with RA PB-differentiated macrophages and normal monocytes (Fig. 2A). Consistent with our mRNA results, FACS

analysis demonstrated that percent TLR5 was significantly reduced both in RA and NLs when monocytes were differentiated into macrophages; however, percent TLR5 was 4-fold higher in RA monocytes and macrophages compared with NL counterpart cells (Fig. 2B, 2C). Despite reduction of TLR5 expression during monocyte to macrophage differentiation, TLR5 expression is significantly increased in macrophages isolated from RA joints, compared with control or RA PB macrophages. Altogether, our results suggest that RA ST and fluid macrophages as well as RA PB monocytes may be an important source for TLR5 response.

### TLR5 ligation can modulate synovial fluid-induced TNF-α transcription in RA monocytes and expression of TLR5 on these cells strongly correlates with DAS28 and TNF-α

Because expression of TLR5 was higher in RA PB monocytes compared with differentiated macrophages, we asked whether ligation of TLR5 in RA monocytes may affect disease pathogenesis. Previous studies have identified a number of endogenous TLR2 and/or TLR4 ligands in RA synovial fluid (33, 34). Hence, synovial fluid-mediated TNF-α transcription in RA monocytes was examined to determine whether endogenous TLR5 ligand(s) were present in RA synovial fluid. Our results show that blockade of TLR5 on RA monocytes greatly downregulates (5-fold decrease; 80% reduction) TNF-α transcription activated by RA synovial fluid (Fig. 3A), suggesting that ligation of TLR5 by potential endogenous ligands expressed in RA synovial fluid may be partially responsible for joint TNF-α modulation. We further validated that the inhibitory effect of anti-TLR5 Ab on RA synovial fluid-mediated TNF-α was specifically due to blockade of TLR5 ligation and had no effect on cell necrosis (Fig. 3B). Given that ligation of TLR5 plays a role in joint TNF-α regulation, we asked whether expression of these two factors correlate with each other
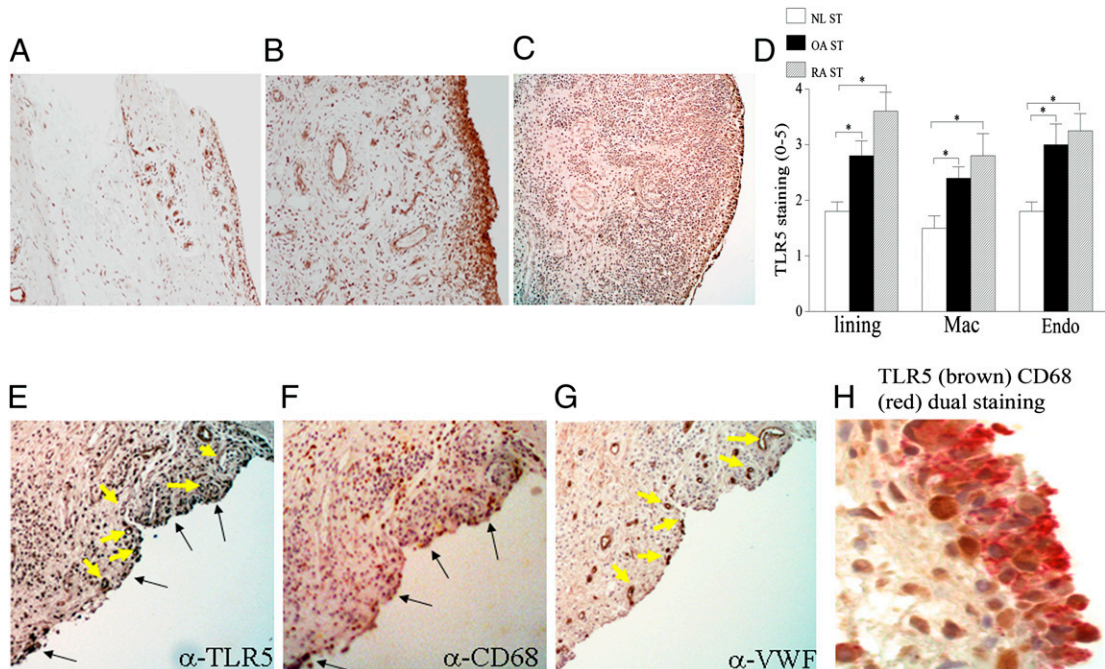


**FIGURE 1.** TLR5 expression is increased in RA and OA ST lining and sublining macrophages and endothelial cells compared with NL ST. NL (**A**), OA (**B**), and RA (**C**) ST were stained with anti-human TLR5 (A–C) (original magnification ×200), and positive immunostaining was scored on a 0–5 scale (**D**). ST lining and sublining macrophage (Mac) and endothelial (Endo) immunostaining are shown as mean ± SEM; ($n = 5$–7). *$p < 0.05$. RA serial sections were stained with anti-TLR5 (**E**), anti-CD68 (**F**), and anti-VWF (**G**) Abs to distinguish TLR5 immunostaining on RA ST macrophages and endothelial cells (original magnification ×400). Black (E, F) and yellow arrows (E, G) demonstrate colocalization of TLR5 on CD68^{+} and VWF^{+} cells. (**H**) RA ST were stained for anti-TLR5 (brown staining) and anti-CD68 Abs (fast red staining) (original magnification ×800) to demonstrate TLR5 costaining on lining macrophages.
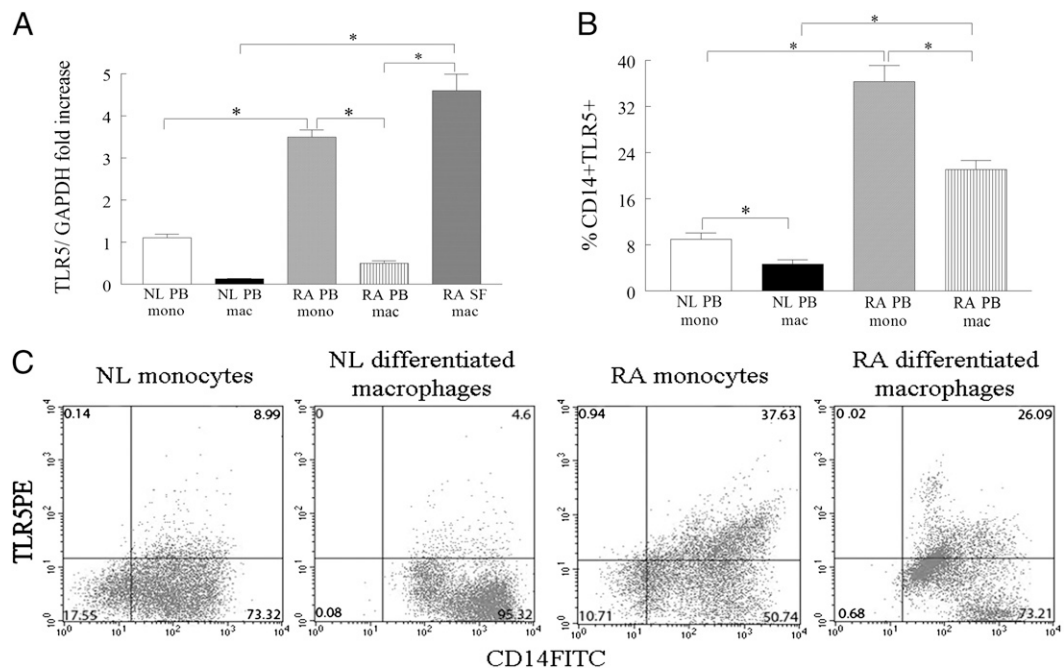
**FIGURE 2.** TLR5 is upregulated in RA synovial fluid (SF) compared with RA and NL PB macrophages. (**A**) TLR5 mRNA levels were determined in NL (*n* for monocytes [mono] or macrophages [mac] = 11 or 18) and RA PB monocytes (*n* = 11) and differentiated macrophages (*n* = 15) as well as in RA SF macrophages (*n* = 10) by using real-time RT-PCR. The data are shown as fold increase above NL PB monocytes and are normalized to GAPDH. (**B**) Normal and RA PB monocytes and differentiated macrophages were immunostained with CD14 labeled with FITC and TLR5 conjugated with PE to determine percent TLR5$^+$ cells (*n* = 6–10). The values are presented as mean ± SEM of percent CD14$^+$TLR5$^+$ in each cell population. (**C**) Representative flow cytometry histograms showing CD14$^+$TLR5$^+$ in NL and RA PB monocytes and differentiated macrophages. *$p < 0.05$.

and/or DAS28. We found that the levels of TLR5 and TNF-α in RA monocytes were closely related ($R^2 = 0.71$; $p = 4.62 \times 10^{-14}$) (Fig. 3C). Furthermore, data analyzed by regression analysis demonstrated that patients with greater levels of DAS28 had increased expression of TLR5 ($R^2 = 0.57$; $p = 1.88 \times 10^{-9}$) (Fig. 3D) and TNF-α ($R^2 = 0.62$; $p = 1.34 \times 10^{-10}$) (Fig. 3E) in RA
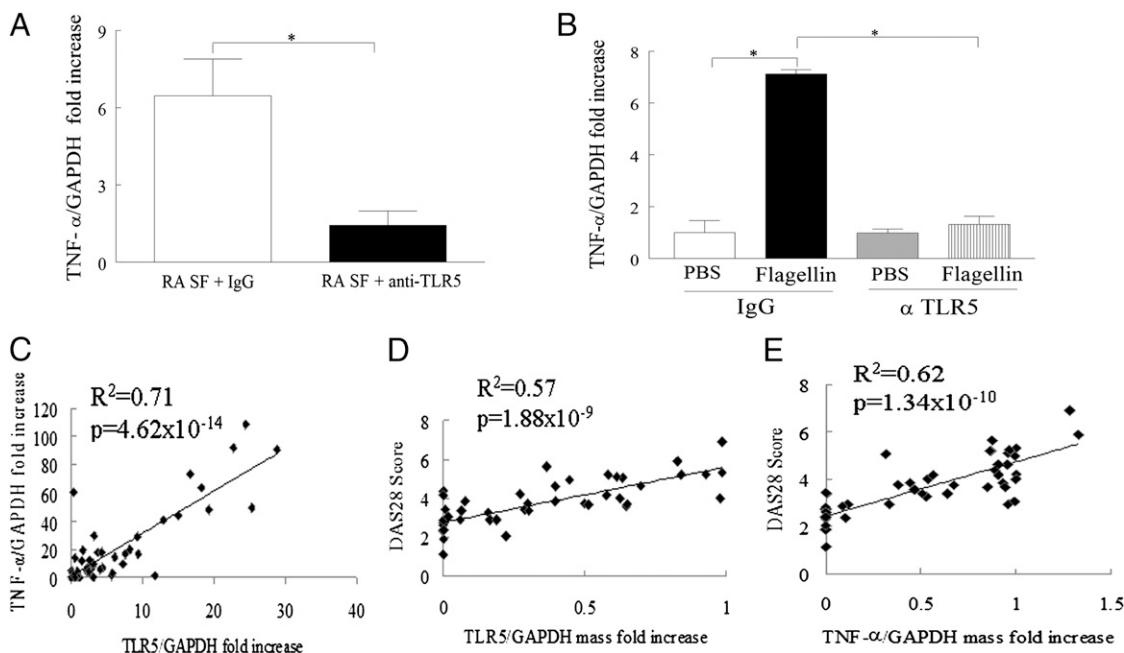


**FIGURE 3.** TLR5 ligation can regulate synovial fluid (SF)-induced TNF-α transcription in RA monocytes and expression of TLR5 on these cells strongly correlates with DAS28 and TNF-α levels. (**A**) RA monocyte from six different patients were treated with anti-TLR5 Ab or IgG (10 μg/ml; InvivoGen) for 1 h prior to being treated with RA SF (10%; *n* = 6) for 6 h. (**B**) RA monocytes were pretreated with anti-TLR5 Ab or IgG control 1 h prior to treating the cells with PBS or flagellin for 6 h. Subsequently, the TNF-α mRNA levels were quantified in (A) and (B) by real-time RT-PCR and normalized to GAPDH value. In (A), the data are shown as fold increase above RA monocytes treated with RA SF plus anti-TLR5 Ab. Whereas in (B), the data are shown as fold increase above the IgG-pretreated PBS group. Linear regression analysis was used to compare TNF-α levels with TLR5 (**C**) (*n* = 48 RA patients) as well as DAS28 score with expression of TLR5 (RNA mass normalized to GAPDH mass) (**D**) (*n* = 45 RA patients) or TNF-α (RNA mass normalized to GAPDH mass) (**E**) (*n* = 45 RA patients) in RA monocytes. The mRNA expression in RA monocytes is shown as a fold increase above NL PB monocytes and is normalized to GAPDH. *$p < 0.05$.
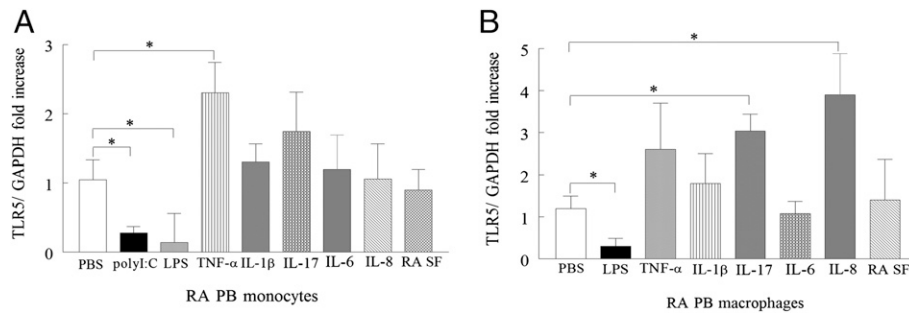
**FIGURE 4.** Proinflammatory factors induce the expression of TLR5 in RA PB monocytes and in vitro-differentiated macrophages. RA PB monocytes (**A**) or in vitro-differentiated macrophages (**B**) were untreated (PBS) or treated with poly I:C (10 ng/ml; only in RA monocytes) LPS (10 ng/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-17 (50 ng/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), or RA synovial fluid (SF) (10%) for 6 h, and expression of TLR5 was measured by real-time RT-PCR (n = 5–12). The data are shown as fold increase above untreated RA PB monocytes or macrophages and are normalized to GAPDH. Values demonstrate mean ± SEM. *p < 0.05.

monocytes. These results suggest that RA disease expression is related to ligation of TLR5 and production of TNF-α from RA monocytes.

*Proinflammatory factors regulate expression of TLR5 in RA monocytes and macrophages*

To determine which factors modulate expression of TLR5 in RA PB monocytes or in vitro-differentiated macrophages, cells were either untreated or treated with poly I:C (only in RA monocytes), LPS, IL-1β, TNF-α, IL-17, IL-6, IL-8, or RA synovial fluid. Results from these experiments demonstrate TLR5 expression was modulated by TNF-α in RA monocytes and by IL-17 and IL-8 in RA macrophages; however, expression levels of TLR5 were suppressed by TLR3 and TLR4 ligation in RA monocytes and/or differentiated macrophages (Fig. 4). Hence, the data suggest that with the exception of LPS, the expression of TLR5 in RA

monocytes and macrophages is differentially regulated in RA monocytes and differentiated macrophages.

*Ligation of TLR5 induces production of proinflammatory factors in RA PB monocytes and macrophages*

Next, we asked whether RA monocytes and differentiated macrophages respond to ligation of flagellin to TLR5. For this purpose, RA monocytes and differentiated macrophages were activated with different doses of flagellin, and cells were screened for transcription (6 h) and production (24 h) of proinflammatory factors such as TNF-α, IL-6, and CCL2. Generally, transcription but not the secretion of TNF-α, IL-6, and CCL2 was dose dependently increased with flagellin stimulation in RA monocytes and differentiated macrophages (Figs. 5, 6A–C). Although TLR5 expression was greatly elevated in RA monocytes compared with RA-differentiated macrophages, TLR5 ligation resulted in higher
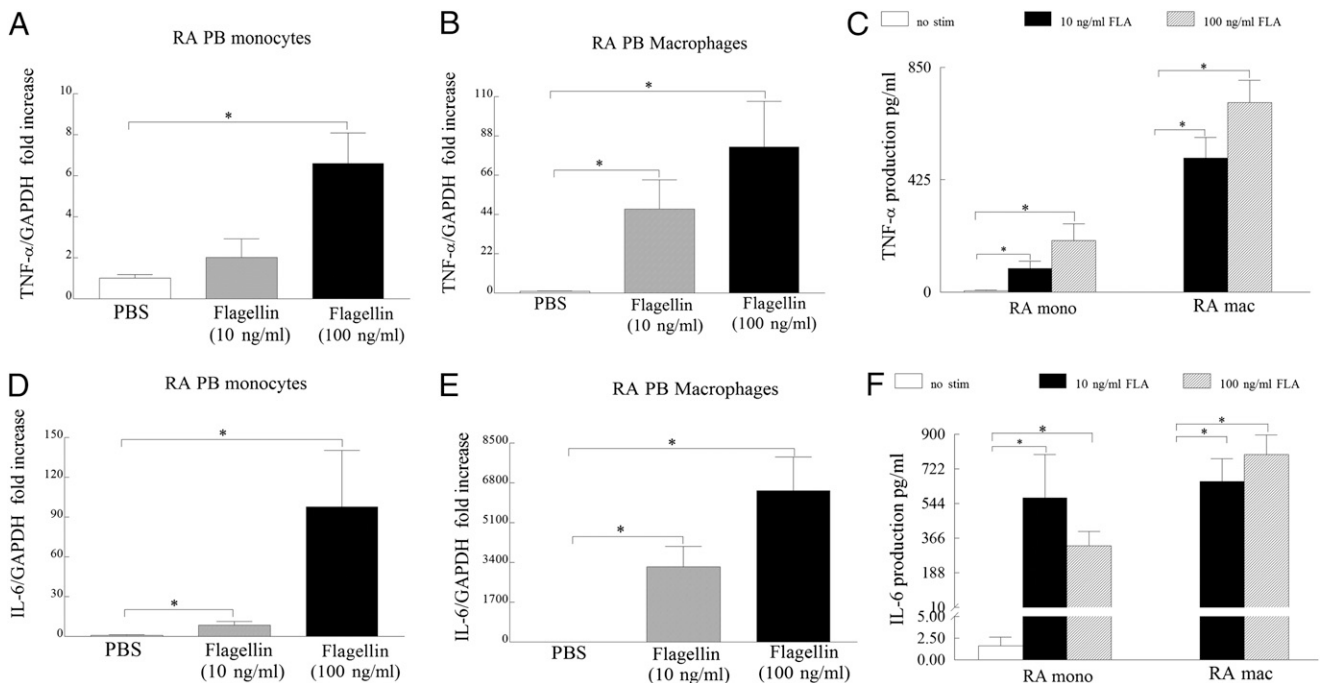


**FIGURE 5.** TLR5 ligation induces expression and production of TNF-α and IL-6 in RA monocytes and macrophages. RA monocytes (**A**, **D**) and differentiated macrophages (**B**, **E**) were either untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 6 h, and expression levels of TNF-α (A, B) and IL-6 (D, E) were quantified by real-time RT-PCR, n = 6–10. The data are shown as fold increase above untreated cells and were normalized to GAPDH values. Supernatants were harvested from RA monocytes (mono) or differentiated macrophages (mac) untreated (PBS) or treated with flagellin 10 or 100 ng/ml for 24 h, and TNF-α (**C**) and IL-6 (**F**) levels were determined by ELISA; n = 5–8. Values demonstrate mean ± SEM. *p < 0.05.

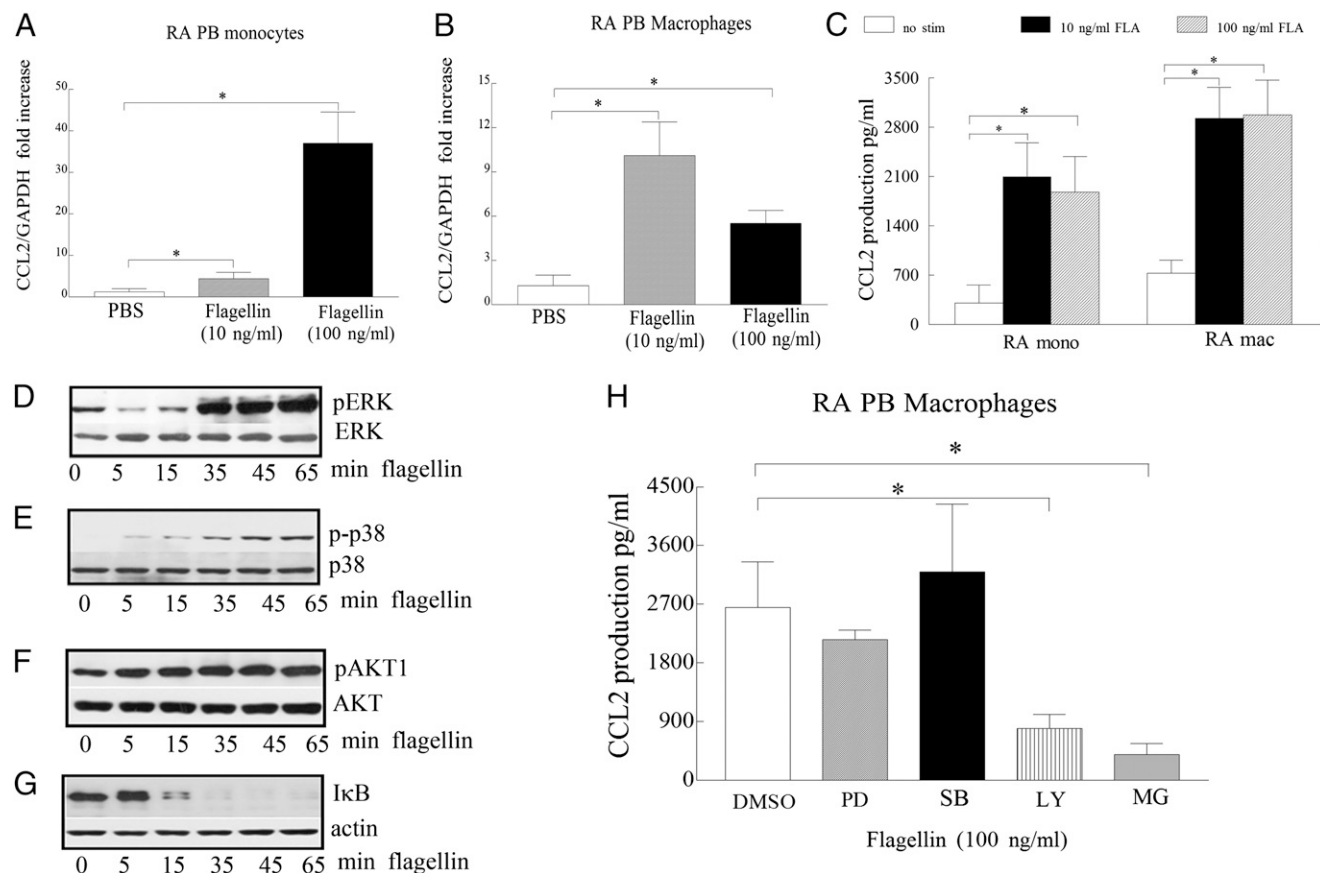**FIGURE 6.** CCL2 levels are increased following TLR5 ligation in RA monocytes and macrophages. Furthermore, in RA macrophages, flagellin-induced CCL2 production is modulated by PI3K and NF-κB pathways. RA monocytes (**A**) and differentiated macrophages (**B**) were either untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 6 h, and expression levels of CCL2 (A, B) were quantified by real-time RT-PCR; $n = 6$–10. The data are shown as fold increase above untreated cells and were normalized to GAPDH. Supernatants were harvested from RA monocytes (mono) or differentiated macrophages (mac) untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 24 h, and CCL2 (**C**) levels were determined by ELISA. Values are the mean ± SEM; $n = 5$–8. To determine the mechanism of TLR5 activation in RA macrophages, cells were stimulated with flagellin at 100 ng/ml for 0–65 min, and the cell lysates were probed for, p-ERK (**D**), p-p38 (**E**), p-AKT1 (**F**), and degradation of IκB (**G**) and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA macrophages, cells were untreated (DMSO) or treated with 10 μM inhibitors to ERK (PD98059), p38 (SB203580), PI3K (LY294002), or NF-κB (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA (**H**). Values are the mean ± SEM; $n = 4$. *$p < 0.05$.

(TNF-α) or comparable production of proinflammatory factors (IL-6 and CCL2) in RA macrophages compared with that of monocytes. These results suggest that despite lower expression of TLR5 in RA macrophages, both monocytes and macrophages respond comparably to TLR5 ligation.

*Flagellin-induced CCL2 is regulated by NF-κB and PI3K pathways in RA macrophages*

We next inhibited flagellin-activated pathways in RA-differentiated macrophages to determine signaling pathways contributing to flagellin-mediated proinflammatory factor production. We found that p38 (5 min), AKT1 (5 min), ERK (35 min), and NF-κB (15 min) pathways (Fig. 6D–G) were activated by flagellin stimulation in RA-differentiated macrophages. We chose to examine the regulation of flagellin-induced CCL2 because this chemokine was detected both in RA-differentiated macrophages and fibroblasts. Although chemical inhibitors to NF-κB and PI3K suppressed flagellin-induced CCL2 secretion by 3- to 6-fold ($p < 0.05$; Fig. 6H), inhibition of p38 or ERK pathway did not reduce the levels of CCL2 secretion by RA-differentiated macrophages. Our results suggest that activation of NF-κB and PI3K by flagellin regulates CCL2 production in RA-differentiated macrophages.

*TLR5 is elevated in RA fibroblasts and its expression is responsive to stimulation*

On the basis of our histological data, we asked whether expression of TLR5 was elevated in RA compared with NL ST fibroblasts. Results obtained from real-time RT-PCR demonstrate that TLR5 (Fig. 7A) expression was 23-fold greater in RA compared with NL ST fibroblasts. We next show that with the exception of LPS, all other proinflammatory factors such as TNF-α (14-fold), IL-1β (7-fold), IL-17 (47-fold), IL-6 (31-fold), IL-8 (20-fold), and RA synovial fluid (28-fold) greatly upregulate the expression of TLR5 in RA fibroblasts (Fig. 7B). To determine whether RA fibroblasts respond to TLR5 ligation, flagellin-activated cells (at two different doses) were screened for a variety of proinflammatory factors. Unlike RA monocytes and differentiated macrophages that are very responsive to flagellin stimulation, RA fibroblasts produce increased levels of IL-6 and CCL2 only when activated with a higher dose of flagellin (100 ng/ml). We found that flagellin activates JNK (15min), ERK (15 min), AKT1 (35min), and NF-κB (35 min) pathways in RA fibroblasts (Fig. 7D–G). Both in RA fibroblasts and macrophages, ligation of TLR5-induced CCL2 production was modulated by NF-κB and PI3K activation (Figs. 6H, 7H). These data suggest that although
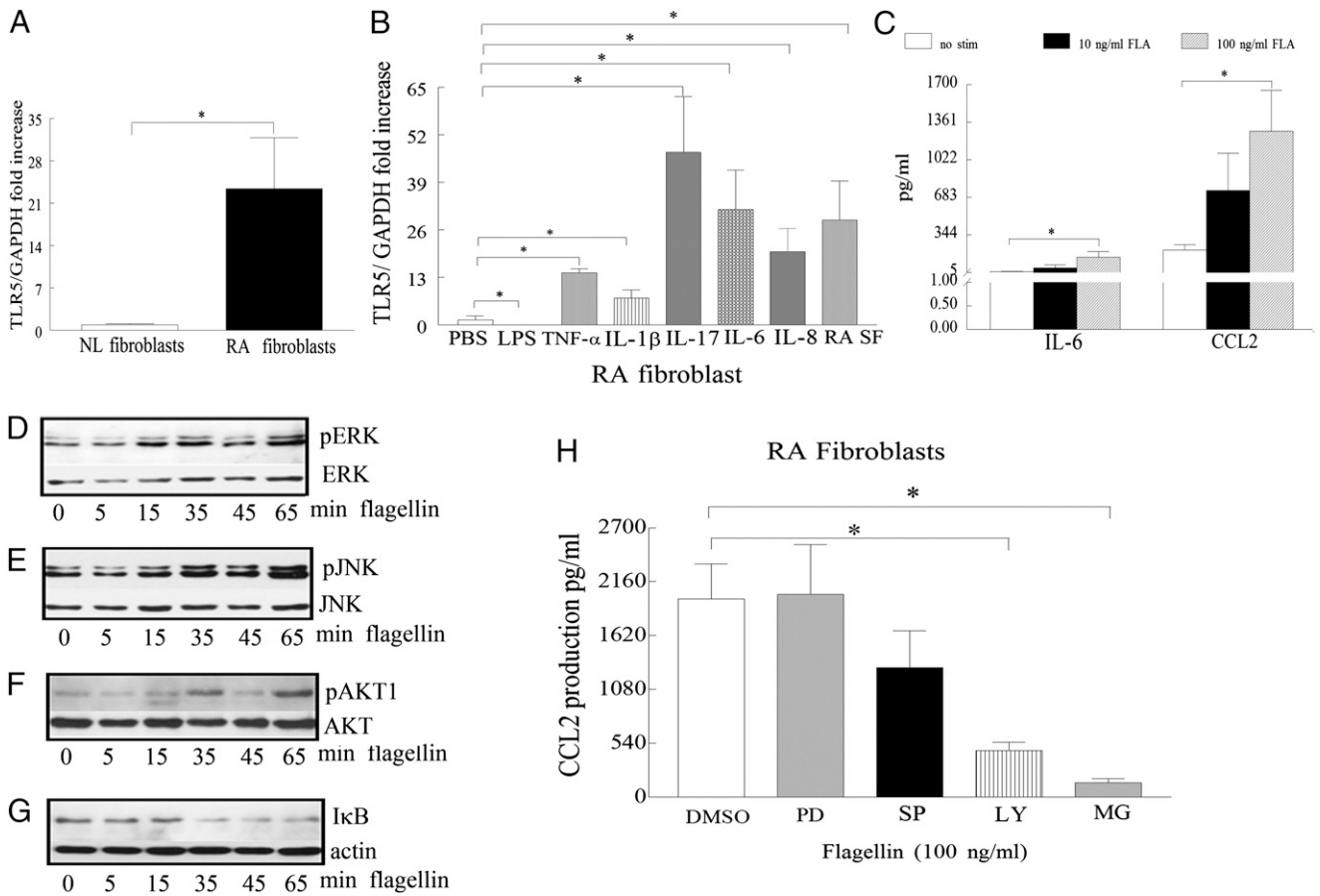
**FIGURE 7.** Expression of TLR5 in RA ST fibroblasts is very responsive to stimulation; however, only higher concentrations of flagellin can induce production of IL-6 and CCL2, which is modulated by PI3K and NF-κB pathways. (**A**) TLR5 mRNA levels were determined in NL and RA ST fibroblasts using real-time RT-PCR ($n = 7$). The data are shown as fold increase above NL ST fibroblasts and are normalized to GAPDH. (**B**) RA ST fibroblasts were untreated (PBS) or treated with LPS (10 ng/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-17 (50 ng/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), or RA SF (10%) for 6 h, and the expression of TLR5 was measured by real-time RT-PCR ($n = 5–12$). The data are shown as fold increase above untreated RA fibroblasts and are normalized to GAPDH. (**C**) Supernatants were harvested from RA fibroblasts untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 24 h, and IL-6 and CCL2 levels were determined by ELISA ($n = 4$). Values demonstrate mean ± SEM; *$p < 0.05$. To determine the mechanism of TLR5 activation in RA fibroblasts, cells were stimulated with flagellin 100 ng/ml for 0–65 min, and the cell lysates were probed for p-ERK (**D**), p-JNK (**E**), p-AKT1 (**F**), and degradation of IκB (**G**) and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA fibroblasts, cells were untreated (DMSO) or treated with 10 μM inhibitors to ERK (PD98059), JNK (SP600125), PI3K (LY294002), or NF-κB (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA (**H**). Values are the mean ± SEM; $n = 4$. *$p < 0.05$.

ligation of TLR5 can induce production of proinflammatory factors through the same signaling pathways in both RA macrophages and fibroblasts, macrophages are comparatively more sensitive to TLR5 activation.

## Discussion

In the current study, we show that RA and OA ST lining and sublining macrophages and endothelial cells express higher levels of TLR5 than tissues of normal controls. We found that transcription levels of TLR5 were elevated in RA synovial fluid macrophages and RA monocytes compared with RA and NL-differentiated macrophages. Confirming histological studies, TLR5 levels were also elevated in RA compared with NL fibroblasts. We show that in RA fibroblasts and macrophages, the TLR5 mRNA concentration was modulated by IL-17 and IL-8. Despite elevated cell surface levels of TLR5 in RA PB monocytes compared with differentiated macrophages, production of proinflammatory factors was comparable in both cell types, which was higher than what was secreted by RA fibroblasts following ligation. Most importantly, we document that in RA monocytes, TLR5 is

a regulator of synovial fluid-mediated TNF-α transcription, and levels of this receptor are strongly correlated to TNF-α and DAS28 score. These results suggest that TLR5 endogenous ligand (s) in the RA joint may potentially activate TLR5+ RA monocytes and contribute to production of joint TNF-α and perpetuation of disease activity.

To our knowledge, we show for the first time that TLR5 expression is elevated in RA and OA ST lining and sublining macrophages and endothelial cells compared with normal individuals. However, expression of TLR5 has not been associated with systemic lupus erythematosus (35). Previous studies demonstrate that TLR5 is expressed in dendritic cells (36), neutrophils (37), and synovial fibroblasts from patients with juvenile idiopathic arthritis (38) and in a number of endothelial cell lines (39) however its expression is undefined in RA ST and blood cells.

Interestingly, we found that differentiation of RA monocytes to macrophages reduces TLR5 expression, as confirmed by both real-time RT-PCR and FACS studies. The same trend was also observed in normal cells. As with TLR5, expression of TLR2 was greater in normal monocytes compared with PB-differentiated macrophages,

whereas similar levels of TLR4 were detected in normal PB monocytes and differentiated macrophages (7). Furthermore, elevated expression levels of TLR2 and TLR4 in RA synovial fluid macrophages compared with normal macrophages (7) is consistent with our findings with TLR5. In contrast to our results, others have shown that TLR5 is similarly expressed in normal PB monocytes and macrophages (37). The discrepancy in the data may be due to monocyte isolation technique as well as using 100 ng/ml M-CSF for macrophage differentiation studies (37). In addition, we demonstrate that TLR3 and TLR4 ligation reduced TLR5 expression on RA monocytes and/or macrophages or fibroblasts. TRIF is an adaptor protein that is shown to degrade TLR5 expression through a caspase-dependent manner (40). Hence, suppression of TLR5 expression in RA cells may be due to activation of TRIF by TLR3 or TLR4 ligation. In RA fibroblasts, although expression of TLR5 is reduced by TLR4 ligation, stimulation with IL-1β has a reverse effect, and this may be due to its lack of association with the TRIF pathway (41). With the exception of LPS, TLR5 expression is differentially regulated in monocytes and macrophages. Others have shown that in human monocytes, expression of TLR5 is suppressed by TLR2 ligation as well as stimulation with IFN-γ and GM-CSF; however, TLR5 expression is greatly increased by flagellin ligation (37, 42). In RA macrophages and fibroblasts, expression of TLR5 was modulated by IL-17. Previous studies have shown that TLR5 ligation can induce Th17 cell differentiation in normal PB mononuclear cells (43) as well as the production of IL-17 in splenocytes (44). IL-17 can also enhance TLR5-induced TNF-α and IL-1β production in epithelial cells (45). These results suggest that expression and ligation of TLR5 on cells present in RA ST lining may be in feedback regulation with Th17 cell differentiation and production of joint IL-17.

Our results suggest that TLR5 endogenous ligand(s) may be present in synovial fluid because blockade of this receptor on monocytes significantly reduces TNF-α transcription induced by synovial fluid. Interestingly, a number of endogenous TLR ligands have been identified in RA ST and fluid including fibrinogen, heat shock protein (HSP)60, 70, and 96 and EDA fibronectin that bind to TLR2 and/or TLR4 (33, 34). Previous studies demonstrate that TLR5-transfected reporter HEK 293T cells stimulated with full-length HSP70 had enhanced flagellin-induced NF-κB–mediated luciferase activity; however, this effect was not detected with HSP70 treatment alone (46). These findings suggest that HSP70 expressed in RA synovial fluid (47), ST macrophages, and fibroblasts (33) may be a chaperone protein for TLR5 endogenous ligand(s) (48). Lectins have also been identified as novel agonists for cell surface-bound TLRs (49). On the basis of earlier investigations, there is a possibility that HSPs (33, 34) and/or lectins (49) may be potential TLR5 endogenous ligands in RA joints. Therefore, studies are currently being conducted to identify RA synovial fluid TLR5 endogenous ligands (not within the scope of this study).

Our results suggest that ligation of synovial fluid TLR5 endogenous ligands to TLR5$^+$ monocytes can contribute to production of joint TNF-α, which in turn can further upregulate expression of TLR5 on these cells. Once RA monocytes reach their destination in the joint and differentiate to macrophages, TLR5 expression is no longer modulated by TNF-α, and their levels are reduced; however, they remain at least as responsive to ligation as RA monocytes. Perhaps in RA monocytes, TLR5 levels correlate with DAS28 and TNF-α and are in a feedback regulation with TNF-α by producing and responding to this factor to perpetuate disease.

When RA monocytes and differentiated macrophages were stimulated with flagellin, similar levels of IL-6 and CCL2 were produced despite RA macrophages having lower TLR5 expression compared with RA monocytes. This may be due to monocytes being in circulation, whereas macrophages are immobilized in the inflammatory milieu of RA ST in cell-to-cell contact with other macrophages or RA fibroblasts, therefore amplifying the activation response. It is also possible that macrophages from RA ST, like those from RA synovial fluid, have higher TLR5 expression compared with RA monocytes, and maybe the presence of proinflammatory factors is required to enhance TLR5 expression during the differentiation process, which is available in the RA joint and unavailable in the culture system. In contrast to our results, other studies were unable to detect TNF-α production when normal monocytes were activated with flagellin (50). This may be due to lower levels of TLR5 expression in normal cells compared with RA monocytes as well as isolation and culturing methods. However, consistent with our data, they were able to demonstrate high levels of CCL2 following TLR5 ligation in normal monocytes (50).

Unlike RA fibroblasts where only higher concentrations of flagellin (100 ng/ml) are capable of inducing expression of IL-6 and CCL2, in RA monocytes and macrophages, ligation of TLR5 with lower concentrations (10 ng/ml) can produce these factors. Conversely, in skeletal muscle cells, ligation of TLR5 was unable to produce significant levels of CCL2 without IFN-γ priming (51). Despite activation of nonoverlapping pathways in RA fibroblasts and macrophages by TLR5 ligation, CCL2 production was modulated by inhibition of NF-κB and PI3K in both cell types. In contrast to our results, blockade of PI3K or use of PI3K/AKT-deficient mice resulted in marked increase in flagellin-induced IL-6 or IL-8/KC levels (52), indicating that the proinflammatory factors produced as a result of TLR5 ligation are differentially regulated in mice and humans.

In conclusion, to our knowledge, we demonstrate for the first time that TLR5 is expressed in RA ST macrophages and fibroblasts as well as RA PB monocytes. We further document modulating factors and pathways contributing to TLR5 inflammatory response. Moreover, our study highlights that there is a strong correlation between TNF-α and TLR5 expression with disease activity in RA monocytes suggesting that TLR5 may be a TNF-α responsive gene that is linked to RA progression.

## Disclosures
The authors have no financial conflicts of interest.

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